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IMPROVED METHOD FOR THE PREVENTION OR REDUCTION OF HAZE IN BEVERAGES

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IMPROVED METHOD FOR THE PREVENTION OR REDUCTION OF HAZE IN BEVERAGES

5 The invention relates to a method for the prevention or reduction of haze in a beverage by the addition of an endoprotease and to new beverages obtainable by the method according to the invention. It also relates to new endoproteases.

10 Haze is a well known phenomenon in the beverage industry. Haze can for example be present in beer, wine and fruit juice. Haze formation can occur at different stages during the brewing process. In "Enzymes in food processing" edited by T. Nagodawithana and G. Reed, 3rd edition, Academic press Inc., San Diego, Chapter V, p.448-449, it has been proposed that the haze in beer is the result of interactions between beer proteins and polyphenolic procyanidins. It is explained that in beer haze
15 is often formed upon chilling of the beer. Beer is fermented and then matured, often under chilled conditions. To achieve clarity, beer is often filtered while cold. In spite of the filtration, beer often becomes cloudy after it is packaged and distributed to customers and chilled again before serving. Eventually haze is even formed in beer when it is not or no longer chilled and a sediment may develop. Haze formation is
20 undesirable because the cloudiness caused by haze formation resembles cloudiness produced by microbial spoilage, which is undesirable, especially for bright beers.

 In "Industrial Enzymology", 2nd edition, Chapter 2.6, p.124-125, it has been described that haze in beer can result from the cross-linkage of the high molecular weight hordein fraction of malt, containing a high proportion of hydrophobic
25 amino acids, which combines with polyphenols principally consisting of proanthocyanidins and catechins (flavanoids). It is described that small amounts of carbohydrates and trace mineral ions are also involved in haze formation, as well as oxidation, which is stated to play an important part in polymerization of polyphenols to produce irreversible haze. It is proposed that polyphenols combine slowly with protein
30 to form chill haze when cooled, but which redissolve when warmed up. Eventually, however, as polyphenols polymerize and increase in size they become insoluble at room temperature to form irreversible or permanent haze.

 In several other publications it has been proposed that the formation of haze in for example beer, wine and fruit juice, coffees and teas is the result of
35 interactions between proteins and polyphenols (K.J. Siebert et al, J. Agric. Food Chem. 44 (1996) 1997-2005 and K.J. Siebert et al, J. Agric. Food Chem. 44 (1996) 80-85, K.J.

Siebert, J. Agric. Food Chem. 47 (1999) 353-362). Since its discovery by L. Wallerstein in 1911, it has been known that a method for the reduction of chill haze formation in beer is the addition of papain to the beer. Papain is an extract of papaya having proteolytic activity. In "Enzymes in food processing" edited by T.

5 Nagodawithana and G. Reed, 3rd edition , Academic press Inc., San Diego, Chapter V, p.448-449 papain is described as being far superior to any other enzyme for the prevention of chill haze in beer. The exact mechanism by which papain works, however, has never been determined ("Enzymes in food processing" edited by T. Nagodawithana and G. Reed, 3rd edition , Academic press Inc., San Diego, Chapter V,
10 p.448-449).

A disadvantage of the use of papain however, is that it has a negative effect on foam. Proteins are necessary to form stable foam on beer. By its proteolytic activity, however, papain adversely affects head foam stability.

Haze formation in wine has been discussed in e.g. "Enzymes in food
15 processing" edited by T. Nagodawithana and G. Reed, 3rd edition , Academic press Inc., San Diego, Chapter 16, p.425, where it is described that grape proteins are held responsible for the formation of haze during the storage of wine. If precipitation is formed in wine after bottling, the wine becomes less attractive to the consumer, which will affect sales. To prevent precipitation, for example bentonite is used. Although
20 bentonite and other adsorbents are successful in removing the proteins, it is not selective and removes other desirable compounds from wine, often affecting the organoleptic properties of wine. In addition, the use of bentonite results in a considerable loss of wine and if the dumping of waste containing bentonite presents difficulties.

25 Although the mechanism is not well understood, it is assumed that the addition of papain hydrolyses the protein in beer to such an extent that a protein-polyphenol haze is not formed or is formed to a smaller extent. Bentonite is used for a similar reason in wine: by absorbing proteins it prevents the formation of protein-polyphenol haze and precipitates. Instead of removing the protein, however,
30 polyphenols may be removed to reduce or prevent haze formation. A typical example of a compound used to remove polyphenols from beverages is polyvinylpolypyrrolidone (PVPP). Lately it has been recognized that polyphenols are important anti-oxidants. Because of all the beneficial effects attributed to anti-oxidants, the option of removing polyphenols from beverages is not the most attractive way to prevent the formation of
35 haze.

Since all known techniques for the prevention or removal of haze

have drawbacks, there is still a need for a new method for the prevention or reduction of haze in beverages.

It is an object of the present invention to provide a method for the prevention or reduction of haze in a beverage.

5 Surprisingly, it has been found that this object is achieved by providing a method for the prevention or reduction of haze in a beverage wherein an endoprotease with an acidic pH optimum is added to the beverage. Preferably this endoprotease is an isolated endoprotease that has prolyl-specific endoprotease activity and/or hydroxyprolyl-specific activity and/or alanine specific activity..

10 In the framework of this invention the term "beverage" includes beverages in all stages of their preparation. Thus, a beverage is not only a beverage ready for consumption but also any composition used to prepare the beverage. For example, wort as used in beer preparation is encompassed by the term "beverage" as used herein. Also, the addition of a prolyl-specific endoprotease during the preparation
15 of a beverage to compositions that are not or not entirely liquid is intended to fall within the method according to the invention. A prolyl-specific endoprotease added to a mash at the start of beer brewing is an example of such a composition.

A prolyl specific endoprotease is defined as a preferably purified endoprotease that cuts proteins or peptides near or at places where the protein or
20 peptide contains a prolyl-residue in its chain. Preferably, in a method according to the invention, a prolyl specific endoprotease is used that cuts proteins or peptides at places where the protein or peptide contains a prolyl-residue.

A hydroxy-prolyl specific endoprotease (endo-hydroxy pro) is defined as a preferably purified endoprotease that cuts proteins or peptides near or at places
25 where the protein or peptide contains a hydroxy-prolyl-residue in its chain. Preferably, in a method according to the invention, a hydroxy-prolyl specific endoprotease is used that cuts proteins or peptides at places where the protein or peptide contains a hydroxy-prolyl-residue.

An alanine specific endoprotease (endo-ala) is defined as a
30 preferably purified endoprotease that cuts proteins or peptides near or at places where the protein or peptide contains an alanine-residue in its chain. Preferably, in a method according to the invention, an alanine-specific endoprotease is used that cuts proteins or peptides at places where the protein or peptide contains an alanine-residue.

In the method according to the invention, a prolyl-specific
35 endoprotease is even more preferably used that cuts prolyl-residues at their C-terminus. A prolyl-specific endoprotease that cuts prolyl-residues at their NH2-terminus

is for example described in a publication in Nature of 15 January 1998, Vol.391, p.301-304.

Further haze reduction in a beverage may be achieved by treatment
5 with a prolyl specific and/or hydroxyprolyl specific and/or alanine specific endoprotease in combination with an auxiliary enzyme.

Under certain conditions and in certain beverages, it may be that a further reduction of haze is desired than the level of haze reduction that is achievable with an endo-pro, endo-hydroxy-pro or endo-ala treatment alone. In such cases it may
10 be beneficial to add an auxiliary proteolytic enzyme before, during or after the endo-pro, endo-hydroxy-pro or endo-ala treatment. Such auxiliary enzymes may be endo-proteases or exo-proteases.

Object of this additional treatment is to further increase the solubility of the peptides that remain after endo-pro, endo-hydroxy-pro or endo-ala treatment
15 and/or to further reduce the interaction of the remaining polypeptides with polyphenols.

This objective is met in that an (exo)peptidase is added to the beverage that is able to remove the carboxy-terminal proline residues from the peptides that remain after endo-pro treatment. Alternatively, or in combination with such an exopeptidase, the peptides that remain after endo-pro treatment may be
20 further solubilised by treatment with an endoprotease. Especially suited for that purpose are enzymes that are able to cleave peptide bonds at the N- or C- terminal position of glycine, alanine, serine, asparagine and glutamine residues.

Also, carboxypeptidases that have activity towards synthetic chromogenic peptides FA-Pro or FA Pro-Pro were found to very useful as auxiliary
25 enzymes.

Auxiliary enzymes with specificities as mentioned above may be commercially obtained or may alternatively be isolated with methods known in the art, for instance with the aid of synthetic chromogenic peptides such as

30 Z-X-AA-pNA (formula 1)

or

FA-AA-X (formula 2)

wherein

pNA = paranitroanilide,

FA = furylacryloyl

35 Z = benzyloxycarbonyl,

AA = amino acid glycine, alanine, serine, asparagines or glutamine and

X = any amino acid

The treatment with these auxiliary enzymes should preferably have no adverse effect on the taste, texture or mouth-feel of the beverage. It is also preferred that these auxiliary enzymes have an acidic pH optimum, preferably below, at
5 or around pH 6.0, 5.0, 4.5 or 4.0 or even more preferred below, at or around pH 3.0.

In the production of most beers, special attention is required when adding (auxiliary) proteolytic enzymes in order not to destroy the ability of the beer to form foam.

In a conventional beer brewing process, cereals are milled and
10 mashed and the resulting mash is filtered to give the wort. The wort is then boiled to inactivate all residual enzymic activities and subsequently used to support yeast growth. After yeast growth and filtering, the beer is lagered. It has been described that in this lagering stage, papain (collupuline) may be added to the beverage in order to prevent haze formation. However, the papaine destroys the foam forming ability of the
15 beer.

Responsible for beer foam is a protein called LTP1 (Lipid Transfer Protein), a 10 kDa barley protein with a very sturdy 3-dimensional structure so that proteases normally present during the mashing stage cannot hydrolyse this molecule. However, during the wort boiling stage, LTP1 unfolds so that it becomes susceptible to
20 enzymatic cleavage. To be active in foam formation, the LTP1 needs a certain minimum size. It is now thought that incubation with Collupuline during lagering (i.e. after wort boiling) destroys LTP1 and thereby the foam forming ability of the beer.

The use of EndoPro during either the mashing or the lagering stage reduces haze formation, but does not destroy or seriously affect LTP1 and therefore
25 the use of endo-pro has no negative effects on foam formation.

In particular, the removal of a carboxy-terminal proline residue from the peptides that resulted from the digestion with endo-pro, resulted in an additional reduction of haze in beer. Remarkably good results in this respect were obtained when a proline-specific carboxypeptidase isolated from Xanthomonas was used in
30 combination with endo-pro.

Milk-clotting enzymes like Fromase ® are also very well suited as auxiliary enzyme in the reduction or prevention of haze when used in combination with endo-pro. Fromase ® is well suited for this purpose since it does not affect LPT1 and therewith preserves the foam forming ability of the beer.

35 Fromase is a commercial product (DSM Food Specialities) obtained from Rhizomucor miehei that is used in cheese production. Fromase is a so-called

aspartic protease (EC 3.4.23). These enzymes are characterised by very low pH optima and an outspoken preference for cleaving peptide bonds between bulky, hydrophobic amino acid residues such as Phe-Phe, Phe-Tyr and Leu-Tyr. Other aspartic acid proteases are pepsin, cathepsin and the various acid proteases from different fungi.

Further haze reduction in wine may also be achieved by additional treatment with auxiliary enzymes in combination with endo-pro, endo-hydroxy-pro or endo-ala hydrolysis.

A protein called chitinase is known to be the main cause of haze problems in wine. This chitinase originates from the grape and is rich in glycine, alanine and serine residues. Since wine has a very low pH, we tried to hydrolyse this chitinase with a complex mixture of acidic endoproteases. Addition of high concentrations of a product called AP 50.000 from Shin Nihon, Japan had no effect on haze formation in wine.

Incubation of wine (white and red) with endo-pro is described in example 7. A further reduction of the haze in wine could be obtained by using auxiliary enzymes such as an exopeptidase that removed the carboxy-terminal proline residues and/or an endoprotease with specificity for glycine.

An additional advantage of using endo-pro in wine (or any other beverage with a very low pH) is that the endo-pro used in the experiments of example 7 is also capable of cleaving peptide bonds after alanine residues as well as a hydroxy-proline residues under acidic conditions (example 13).

In this text, the terms endo-pro, prolyl-specific endoprotease, proline-specific endoprotease, proline-specific endopeptidase and peptide having a prolyl-specific activity or similar expressions are used interchangeably.

In this text, the words peptide and protein are used interchangeably. In this text, the words "haze", "cloudiness" and "turbidity" are also used interchangeably. To quantify the amount of haze in a beverage, a turbidimeter is often used. In a turbidimeter the amount of light is measured that is scattered at a prediscrined angle relative to the direction of the incident light beam. Turbidity measurements are very suitable for the measurement of haze formed as the result of protein-polyphenol interactions.

A polyphenol is defined as a compound having a chemical structure which structure contains at least two aromatic rings substituted with at least one hydroxyl group or having a chemical structure which contains at least one aromatic ring substituted with at least two hydroxyl groups.

Examples of polyphenols are tannins and flavonoids, which include for example catechins, flavonols and anthocyanins.

Endoproteases having a prolyl-specific activity are known (E.C. 3.4.21.26). However, the use of prolyl-specific endoproteases for the prevention or
5 reduction of haze in beverages has never been described or suggested.

As is typical for enzyme activities, the activity of prolyl-specific endoproteases is dependent on the pH. In a preferred embodiment of the method according to the invention, an endoprotease is added to the beverage having a maximum prolyl specific activity at a pH which corresponds to the pH of the beverage it
10 is added to. Preferred beverages are protein containing beverages. In another preferred embodiment, the beverage contains proteins and polyphenols. Preferred beverages are beverages having a pH value below 7.

The method according to the invention is advantageously applied to beer, wine and fruit juice. It may also advantageously be applied to alcoholic beverages
15 other than beer and wine.

The term "beer" as used herein is intended to cover at least beer prepared from mashes prepared from unmalted cereals as well as all mashes prepared from malted cereals, and all mashes prepared from a mixture of malted and unmalted cereals. The term "beer" also covers beers prepared with adjuncts, and beers with all
20 possible alcohol contents.

Fruit juice may be juice obtained from for example red berries, strawberries, apples, pears, tomatoes, citrus fruits, vegetables etc.

The amount of proline-specific endoprotease that is added to a beverage in the method according to the invention may vary between wide limits. In a
25 preferred embodiment of the method according to the invention at least 150 milli-units of proline-specific endoprotease activity, whereby the activity was determined by an activity measurements using Z-Gly-Pro-pNA as a substrate, per gram protein in the beverage is added.

More preferably, at least 500 milli-units of proline-specific
30 endoprotease is added to the beverage, and most preferably, at least 1 unit of proline-specific endoprotease is added.

A maximum amount of proline-specific endoprotease activity to be added cannot be specified. The maximum amount is for example dependent on the desired amount of haze reduction or prevention, the composition of the beverage, the
35 pH of the beverage and the pH at which the endoprotease has its maximum activity.

A prolyl-specific endoprotease may be added at different stages

during the preparation of a beverage.

During the preparation process of beer, the prolyl-specific endoprotease is advantageously added to a mash. In another embodiment, the prolyl-specific endoprotease is added to a fermented beer before haze is formed. However, it is also possible that prolyl-specific endoprotease is added to a fermented beer after haze has been formed. The prolyl-specific endoprotease may advantageously be added to the mashing or maturation step in a process for the preparation of beer.

During the preparation of wine, the prolyl-specific endoprotease is preferably added to a fermented wine. The prolyl-specific endoprotease may advantageously be added after alcoholic fermentation or after malolactic fermentation in a process for the production of a wine.

In a process for the preparation of a fruit juice, the prolyl-specific endoprotease is preferably added during the maceration or depectinization.

Since haze formation often occurs in acidic beverages such as for example beer, wine and fruit juice, prolyl-specific endoproteases having a prolyl specific activity at a pH value below 7 are preferably used. More preferable, prolyl-specific endoproteases having a maximum prolyl specific activity at a pH value below 7.0, or 6.0 are used in the method according to the invention. Most preferred are endoproteases with a pH optimum at or below pH 5.0.

The present invention provides such a peptide, having proline-specific endoprotease activity with a pH optimum around pH 5.0.

The present invention further provides an isolated polypeptide which has proline-specific endoprotease activity selected from the group consisting of:

- (a) a polypeptide which has an amino acid sequence which has at least 40% overall amino acid sequence identity with SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 or a fragment thereof;
- (b) a polypeptide which is encoded by a polynucleotide which hybridizes with
 - (i) the nucleic acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 or a fragment thereof which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, more preferably at least 90% identical over 200 nucleotides, or
 - (ii) a nucleic acid sequence complementary to the nucleic acid sequence of (i)

It also provides a nucleic acid molecule encoding the prolyl-specific endoprotease.

The invention also relates to purified or isolated polypeptides having prolyl-specific endo-protease activity. Preferred are purified prolyl-specific endoproteases having a maximum activity at pH values below 7.

5 The invention provides an isolated polypeptide having the amino acid sequence according to SEQ ID NO: 4, SEQ ID NO: 5 or SEQ ID NO: 7, an amino acid sequence obtainable by expressing the polynucleotide sequences of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, OR SEQ ID NO: 6 in an appropriate host. Also, a peptide or polypeptide comprising a functional equivalent of the above polypeptides is comprised within the present invention. The above polypeptides are collectively comprised in the
10 term "polypeptides according to the invention"

The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than seven
15 amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus. The one-letter code of amino acids used herein is commonly known in the art and can be found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2nd, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring
20 Harbor, NY, 1989)

By "isolated" or "purified" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention as are native or recombinant
25 polypeptides which have been substantially purified by any suitable technique such as, for example, the single-step purification method disclosed in Smith and Johnson, Gene 67:31-40 (1988).

The polypeptide according to the invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium
30 sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

35 Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by

recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

An advantageous embodiment of the invention concerns a purified or isolated polypeptide having endo-pro activity. Such purified or isolated polypeptide may be obtained from a fermentation broth wherein an organism according to the invention, such as an *A. niger* strain carrying a polynucleotide according to the invention, has been cultured. A person skilled in the art will know how to obtain at least partially purified enzyme from the supernatant of such a culture.

A particularly advantageous method of purification is the following. After culturing the cells in an appropriate fermentation broth, the cells were separated from the culture supernatant by centrifugation. The supernatant had a hazy look. Larger particles remaining in the supernatant were then subsequently removed by filtration with 0,5% Dicalite or preferably 1.0% Dicalite in order to prevent clogging of the filter that is applied in the next step. Next, Germ reduction filtration was applied to decrease the amount of germs in the solution. Still, the filtrate was not clear. A Millipore filter with a Molecular weight cut-off value of 10kDalton was subsequently used for further reduction of water, salt and sugar content of the solution. A pressure of 1 bar was applied over the filter. Typical yields obtained were between 50 and 92 % based on units present in the fermentation broth versus units obtained in the purified ultrafiltrate. Typical concentrations of enzyme in the ultrafiltrate result in an prolyl-specific endoprotease activity in the range of 4 to 10 Units per ml.

Further purification was obtained by applying either of the following methods:

Lab-scale purification was performed using the Akta Explorer on a 24 ml Q-sepharose FF column (bed height 12 cm / diameter 1.6 cm). 10 ml UF-concentrate was diluted 10 times in buffer A and applied to the column. Proteins were eluted in a gradient: 0 to 50 % B in 20 CV. Buffer A was 20 mM NaAc pH 5.1. Buffer B was 20 mM NaAc + 1 M NaCl pH 5.1. Flow was 5 ml/min.

Purification was performed using the Akta purifier according to work instruction W-0894.A on a 500 ml Q-sepharose FF column (bed height 23.5 cm / diameter 5 cm). 200 ml UF-concentrate was diluted 10 times in buffer A and applied to the column. Proteins were eluted in a gradient: 0 to 40 % B in 20 CV. Buffer A was

20 mM NaAc pH 5.1. Buffer B was 20 mM NaAc + 1 M NaCl pH 5.1. Flow was 10ml/min. Fractions were manually collected.

The obtained product exhibited a single peak on HPSEC and appeared as a single band in SDS PAGE and IEF. It may thus be concluded that prolyl-specific endoprotease may be purified to homogeneity using Q-sepharose FF. Estimated purity was over 90% and specific activity on Z-gly-Pro-pNA was at least 0.094U/mg.

Polypeptides of the invention may be in an isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as isolated. A polypeptide of the invention may also be in a more substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 70%, e.g. more than 80%, 90%, 95%, 98% or 99% of the proteins in the preparation is a polypeptide of the invention.

Polypeptides of the invention may be provided in a form such that they are outside their natural cellular environment. Thus, they may be substantially isolated or purified, as discussed above, or in a cell in which they do not occur in nature, for example a cell of other fungal species, animals, plants or bacteria.

Advantageously, isolated or purified prolyl-specific endoprotease are used in the method according to the invention.

An isolated or purified proline-specific endoprotease according to the invention preferably has at least 10 units of proline specific endoprotease activity per gram of proteinaceous material. These units should be measured using the synthetic peptide Z-Gly-Pro-pNA at 37 degrees C and pH 5, as described in the Methods section.

Proline-specific endoproteases are widely found in animals and plants, but their presence in microorganisms appears to be limited. To date, proline-specific endoprotease have been identified in species of *Aspergillus* (EP 0 522 428), *Flavobacterium* (EP 0 967 285) and *Aeromonas* (J.Biochem.113, 790-796), *Xanthomonas* and *Bacteroides*. Though the proline-specific enzymes from most of these organisms are active around pH 8, the enzymes according to the invention are optimally active at acidic pH some even have a pH optimum around pH 5. The proline-specific endoprotease of the invention may be isolated from one of the above-mentioned microbial species, particularly from a species of *Aspergillus*. Preferably, the proline-specific endoprotease is isolated from a strain of *Aspergillus niger*. More preferably, the proline-specific endoprotease is isolated from an *Aspergillus niger* host

engineered to overexpress a gene encoding a proline-specific endoprotease, although other hosts, such as *E. coli* are suitable expression vectors. For example, the cloning and overproduction of the *Flavobacterium* derived proline-specific endoprotease in, amongst others, *E. coli* has made certain proline-specific endoproteases available in a pure form. An example of such an overproducing construct is provided in the World Journal of Microbiology & Biotechnology, Vol 11, pp 209-212. An *Aspergillus niger* host is preferably used to produce a non-recombinant self-construct utilizing *A. niger* promoters to drive the expression of a gene encoding an *A. niger* proline-specific endoprotease.

In a first embodiment, the present invention provides an isolated polypeptide having an amino acid sequence which has an overall degree of amino acid sequence identity to amino acids of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 (i.e. the polypeptide) of at least about 40%, preferably at least about 50%, preferably at least about 60%, preferably at least about 65%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, still more preferably at least about 95%, and most preferably at least about 97%, and which has proline specific endoprotease activity.

For the purposes of the present invention, the degree of identity between two or more amino acid sequences is determined by BLAST P protein database search program (Altschul et al., 1997, Nucleic Acids Research 25: 3389-3402) with matrix Blosum 62 and an expected threshold of 10.

A polypeptide of the invention may comprise the amino acid sequence set forth in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: or a substantially homologous sequence, or a fragment of either sequence having proline specific endoprotease activity. In general, the naturally occurring amino acid sequence shown in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 is preferred.

The polypeptide of the invention may also comprise a naturally occurring variant or species homologue of the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7.

A variant is a polypeptide that occurs naturally in, for example, fungal, bacterial, yeast or plant cells, the variant having proline specific endoprotease activity and a sequence substantially similar to the protein of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. The term "variants" refers to polypeptides which have the same essential character or basic biological functionality as the proline specific endoprotease of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7, and includes allelic variants. Preferably, a variant polypeptide has at least the same level of proline specific

endoprotease activity as the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. Variants include allelic variants either from the same strain as the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. or from a different strain of the same genus or species.

5 Similarly, a species homologue of the inventive protein is an equivalent protein of similar sequence which is an proline specific endoprotease and occurs naturally in another species of *Aspergillus*.

10 Variants and species homologues can be isolated using the procedures described herein which were used to isolate the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 and performing such procedures on a suitable cell source, for example a bacterial, yeast, fungal or plant cell. Also possible is to use a probe of the invention to probe libraries made from yeast, bacterial, fungal or plant cells in order to obtain clones expressing variants or species homologues of the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. These clones can
15 be manipulated by conventional techniques to generate a polypeptide of the invention which thereafter may be produced by recombinant or synthetic techniques known *per se*.

20 The sequence of the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 and of variants and species homologues can also be modified to provide polypeptides of the invention. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions. The same number of deletions and insertions may also be made. These changes may be made outside regions critical to the function of the polypeptide, as such a modified polypeptide will retain its proline specific endoprotease activity.

25 Polypeptides of the invention include fragments of the above mentioned full length polypeptides and of variants thereof, including fragments of the sequence set out in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. Such fragments will typically retain activity as an proline specific endoprotease. Fragments may be at least 50, 100 or 200 amino acids long or may be this number of amino acids short of
30 the full length sequence shown in SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7.

35 Polypeptides of the invention can, if necessary, be produced by synthetic means although usually they will be made recombinantly as described below. Synthetic polypeptides may be modified, for example, by the addition of histidine residues or a T7 tag to assist their identification or purification, or by the addition of a signal sequence to promote their secretion from a cell.

Thus, the variants sequences may comprise those derived from

strains of *Aspergillus* other than the strain from which the polypeptide of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7 was isolated. Variants can be identified from other *Aspergillus* strains by looking for proline specific endoprotease activity and cloning and sequencing as described herein. Variants may include the deletion, modification or
5 addition of single amino acids or groups of amino acids within the protein sequence, as long as the peptide maintains the basic biological functionality of the proline specific endoprotease of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7.

Amino acid substitutions may be made, for example from 1, 2 or from 3 to 10, 20 or 30 substitutions. The modified polypeptide will generally retain activity as
10 an proline specific endoprotease. Conservative substitutions may be made; such substitutions are well known in the art. Preferably substitutions do not affect the folding or activity of the polypeptide.

Shorter polypeptide sequences are within the scope of the invention. For example, a peptide of at least 50 amino acids or up to 60, 70, 80, 100, 150 or 200
15 amino acids in length is considered to fall within the scope of the invention as long as it demonstrates the basic biological functionality of the proline specific endoprotease of SEQ ID NO: 4, SEQ ID NO: 5, OR SEQ ID NO: 7. In particular, but not exclusively, this aspect of the invention encompasses the situation in which the protein is a fragment of the complete protein sequence.

In a second embodiment, the present invention provides an to isolated polypeptide which has proline specific endoprotease activity, and is encoded by polynucleotides which hybridize or are capable of hybridizing under low stringency conditions, more preferably medium stringency conditions, and most preferably high stringency conditions, with (i) the nucleic acid sequence of SEQ ID NO: 1, SEQ ID NO:
25 2, SEQ ID NO: 3 OR SEQ ID NO: 6 or a nucleic acid fragment comprising at least the c-terminal portion of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6, but having less than all or having bases differing from the bases of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6; or (ii) with a nucleic acid strand complementary to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6.

The term "capable of hybridizing" means that the target
30 polynucleotide of the invention can hybridize to the nucleic acid used as a probe (for example, the nucleotide sequence set forth in SEQ. ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6, or a fragment thereof, or the complement of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6) at a level significantly above
35 background. The invention also includes the polynucleotides that encode the proline specific endoprotease of the invention, as well as nucleotide sequences which are

complementary thereto. The nucleotide sequence may be RNA or DNA, including genomic DNA, synthetic DNA or cDNA. Preferably, the nucleotide sequence is DNA and most preferably, a genomic DNA sequence. Typically, a polynucleotide of the invention comprises a contiguous sequence of nucleotides which is capable of hybridizing under selective conditions to the coding sequence or the complement of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6. Such nucleotides can be synthesized according to methods well known in the art.

A polynucleotide of the invention can hybridize to the coding sequence or the complement of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 at a level significantly above background. Background hybridization may occur, for example, because of other cDNAs present in a cDNA library. The signal level generated by the interaction between a polynucleotide of the invention and the coding sequence or complement of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 is typically at least 10 fold, preferably at least 20 fold, more preferably at least 50 fold, and even more preferably at least 100 fold, as intense as interactions between other polynucleotides and the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6. The intensity of interaction may be measured, for example, by radiolabelling the probe, for example with ^{32}P . Selective hybridization may typically be achieved using conditions of low stringency (0.3M sodium chloride and 0.03M sodium citrate at about 40°C), medium stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 50°C) or high stringency (for example, 0.3M sodium chloride and 0.03M sodium citrate at about 60°C).

Modifications

Polynucleotides of the invention may comprise DNA or RNA. They may be single or double stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides including peptide nucleic acids. A number of different types of modifications to polynucleotides are known in the art. These include a methylphosphonate and phosphorothioate backbones, and addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

It is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any

particular host organism in which the polypeptides of the invention are to be expressed.

The coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may be modified by nucleotide substitutions, for example from 1, 2 or 3 to 10, 25, 50 or 100 substitutions. The polynucleotide of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends. The modified polynucleotide generally encodes a polypeptide which has proline specific endoprotease activity. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as discussed with reference to polypeptides later.

Homologues

A nucleotide sequence which is capable of selectively hybridizing to the complement of the DNA coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 is included in the invention and will generally have at least 50% or 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 over a region of at least 60, preferably at least 100, more preferably at least 200 contiguous nucleotides or most preferably over the full length of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6. Likewise, a nucleotide which encodes an active proline specific endoprotease and which is capable of selectively hybridizing to a fragment of a complement of the DNA coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6, is also embraced by the invention. A C-terminal fragment of the nucleic acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, more preferably at least 90% identical over 200 nucleotides is encompassed by the invention.

Any combination of the above mentioned degrees of identity and minimum sizes may be used to define polynucleotides of the invention, with the more stringent combinations (i.e. higher identity over longer lengths) being preferred. Thus, for example, a polynucleotide which is at least 80% or 90% identical over 60, preferably over 100 nucleotides, forms one aspect of the invention, as does a polynucleotide which is at least 90% identical over 200 nucleotides.

The UWGCG Package provides the BESTFIT program which may be used to calculate identity (for example used on its default settings).

The PILEUP and BLAST N algorithms can also be used to calculate sequence identity or to line up sequences (such as identifying equivalent or corresponding sequences, for example on their default settings).

5 Software for performing BLAST analyses is publicly available through
the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). This
algorithm involves first identifying high scoring sequence pair (HSPs) by identifying
short words of length W in the query sequence that either match or satisfy some
positive-valued threshold score T when aligned with a word of the same length in a
database sequence. T is referred to as the neighborhood word score threshold. These
10 initial neighborhood word hits act as seeds for initiating searches to find HSPs
containing them. The word hits are extended in both directions along each sequence
for as far as the cumulative alignment score can be increased. Extensions for the word
hits in each direction are halted when: the cumulative alignment score falls off by the
quantity X from its maximum achieved value; the cumulative score goes to zero or
15 below, due to the accumulation of one or more negative-scoring residue alignments; or
the end of either sequence is reached. The BLAST algorithm parameters W , T and X
determine the sensitivity and speed of the alignment. The BLAST program uses as
defaults a word length (W) of 11, the BLOSUM62 scoring matrix alignments (B) of 50,
expectation (E) of 10, $M=5$, $N=4$, and a comparison of both strands.

20 The BLAST algorithm performs a statistical analysis of the similarity
between two sequences. One measure of similarity provided by the BLAST algorithm
is the smallest sum probability ($P(N)$), which provides an indication of the probability by
which a match between two nucleotide or amino acid sequences would occur by
chance. For example, a sequence is considered similar to another sequence if the
25 smallest sum probability in comparison of the first sequence to the second sequence is
less than about 1, preferably less than about 0.1, more preferably less than about 0.01,
and most preferably less than about 0.001.

Primers and Probes

30 Polynucleotides of the invention include and may be used as primers,
for example as polymerase chain reaction (PCR) primers, as primers for alternative
amplification reactions, or as probes for example labelled with a revealing label by
conventional means using radioactive or non-radioactive labels, or the polynucleotides
may be cloned into vectors. Such primers, probes and other fragments will be at least
35 15, for example at least 20, 25, 30 or 40 nucleotides in length. They will typically be up
to 40, 50, 60, 70, 100, 150, 200 or 300 nucleotides in length, or even up to a few

nucleotides (such as 5 or 10 nucleotides) short of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6.

In general, primers will be produced by synthetic means, involving a step-wise manufacture of the desired nucleic acid sequence one nucleotide at a time.

5 Techniques for accomplishing this using automated protocols are readily available in the art. Longer polynucleotides will generally be produced using recombinant means, for example using PCR cloning techniques. This will involve making a pair of primers (typically of about 15-30 nucleotides) to amplify the desired region of the proline specific endoprotease to be cloned, bringing the primers into contact with mRNA,

10 cDNA or genomic DNA obtained from a yeast, bacterial, plant, prokaryotic or fungal cell, preferably of an *Aspergillus* strain, performing a polymerase chain reaction under conditions suitable for the amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme

15 recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Such techniques may be used to obtain all or part of the polynucleotides encoding the proline specific endoprotease sequences described herein. Introns, promoter and trailer regions are within the scope of the invention and may also be obtained in an analogous manner (e.g. by recombinant means, PCR or

20 cloning techniques), starting with genomic DNA from a fungal, yeast, bacterial plant or prokaryotic cell.

The polynucleotides or primers may carry a revealing label. Suitable labels include radioisotopes such as ^{32}P or ^{35}S , enzyme labels, or other protein labels such as biotin. Such labels may be added to polynucleotides or primers of the

25 invention and may be detected using techniques known to persons skilled in the art.

Polynucleotides or primers (or fragments thereof) labelled or unlabelled may be used in nucleic acid-based tests for detecting or sequencing an proline specific endoprotease or a variant thereof in a fungal sample. Such detection tests will generally comprise bringing a fungal sample suspected of containing the DNA

30 of interest into contact with a probe comprising a polynucleotide or primer of the invention under hybridizing conditions, and detecting any duplex formed between the probe and nucleic acid in the sample. Detection may be achieved using techniques such as PCR or by immobilizing the probe on a solid support, removing any nucleic acid in the sample which is not hybridized to the probe, and then detecting any nucleic

35 acid which is hybridized to the probe. Alternatively, the sample nucleic acid may be immobilized on a solid support, the probe hybridized and the amount of probe bound to

such a support after the removal of any unbound probe detected.

The probes of the invention may conveniently be packaged in the form of a test kit in a suitable container. In such kits the probe may be bound to a solid support where the assay format for which the kit is designed requires such binding.

5 The kit may also contain suitable reagents for treating the sample to be probed, hybridizing the probe to nucleic acid in the sample, control reagents, instructions, and the like. The probes and polynucleotides of the invention may also be used in microassay.

10 Preferably, the polynucleotide of the invention is obtainable from the same organism as the polypeptide, such as a fungus, in particular a fungus of the genus *Aspergillus*.

The polynucleotides of the invention also include variants of the sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 which encode for a polypeptide having proline specific endoprotease activity. Variants may
15 be formed by additions, substitutions and/or deletions. Such variants of the coding sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may thus encode polypeptides which have the ability to digest a polypeptide chain at the carboxyterminal side of proline.

20 Production of polynucleotides

Polynucleotides which do not have 100% identity with SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 but fall within the scope of the invention can be obtained in a number of ways. Thus, variants of the proline specific endoprotease sequence described herein may be obtained for example, by probing
25 genomic DNA libraries made from a range of organisms, such as those discussed as sources of the polypeptides of the invention. In addition, other fungal, plant or prokaryotic homologues of proline specific endoprotease may be obtained and such homologues and fragments thereof in general will be capable of hybridising to SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6. Such sequences may be
30 obtained by probing cDNA libraries or genomic DNA libraries from other species, and probing such libraries with probes comprising all or part of SEQ ID. 1 under conditions of low, medium to high stringency (as described earlier). Nucleic acid probes comprising all or part of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6 may be used to probe cDNA or genomic libraries from other species, such as those
35 described as sources for the polypeptides of the invention.

Species homologues may also be obtained using degenerate PCR,

which uses primers designed to target sequences within the variants and homologues which encode conserved amino acid sequences. The primers can contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

5 Alternatively, such polynucleotides may be obtained by site directed mutagenesis of the proline specific endoprotease sequences or variants thereof. This may be useful where, for example, silent codon changes to sequences are required to optimize codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be made in order to
10 introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

The invention includes double stranded polynucleotides comprising a polynucleotide of the invention and its complement.

The present invention also provides polynucleotides encoding the
15 polypeptides of the invention described above. Since such polynucleotides will be useful as sequences for recombinant production of polypeptides of the invention, it is not necessary for them to be capable of hybridising to the sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 OR SEQ ID NO: 6, although this will generally be desirable. Otherwise, such polynucleotides may be labelled, used, and made as
20 described above if desired.

Recombinant Polynucleotides.

The invention also provides vectors comprising a polynucleotide of the invention, including cloning and expression vectors, and in another aspect methods
25 of growing, transforming or transfecting such vectors into a suitable host cell, for example under conditions in which expression of a polypeptide of, or encoded by a sequence of, the invention occurs. Provided also are host cells comprising a polynucleotide or vector of the invention wherein the polynucleotide is heterologous to the genome of the host cell. The term "heterologous", usually with respect to the host
30 cell, means that the polynucleotide does not naturally occur in the genome of the host cell or that the polypeptide is not naturally produced by that cell. Preferably, the host cell is a yeast cell, for example a yeast cell of the genus *Kluyveromyces* or *Saccharomyces* or a filamentous fungal cell, for example of the genus *Aspergillus*.

Polynucleotides of the invention can be incorporated into a
35 recombinant replicable vector, for example a cloning or expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus, in a further

embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered
5 from the host cell. Suitable host cells are described below in connection with expression vectors.

Vectors

The vector into which the expression cassette of the invention is
10 inserted may be any vector that may conveniently be subjected to recombinant DNA procedures, and the choice of the vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extra-chromosomal entity, the replication of which is independent of chromosomal replication, such as a plasmid. Alternatively, the vector
15 may be one which, when introduced into a host cell, is integrated into the host cell genome and replicates together with the chromosome(s) into which it has been integrated.

Preferably, when a polynucleotide of the invention is in a vector it is operably linked to a regulatory sequence which is capable of providing for the
20 expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence such as a promoter, enhancer or other expression regulation signal "operably linked" to a coding sequence is positioned in such a way that
25 expression of the coding sequence is achieved under conditions compatible with the control sequences.

The vectors may, for example in the case of plasmid, cosmid, virus or phage vectors, be provided with an origin of replication, optionally a promoter for the expression of the polynucleotide and optionally an enhancer and/or a regulator of the
30 promoter. A terminator sequence may be present, as may be a polyadenylation sequence. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used *in vitro*, for example for the production of RNA or can be used to transfect or transform a host cell.

35 The DNA sequence encoding the polypeptide is preferably introduced into a suitable host as part of an expression construct in which the DNA

sequence is operably linked to expression signals which are capable of directing expression of the DNA sequence in the host cells. For transformation of the suitable host with the expression construct transformation procedures are available which are well known to the skilled person. The expression construct can be used for transformation of the host as part of a vector carrying a selectable marker, or the expression construct is co-transformed as a separate molecule together with the vector carrying a selectable marker. The vectors may contain one or more selectable marker genes.

Preferred selectable markers include but are not limited to those that complement a defect in the host cell or confer resistance to a drug. They include for example versatile marker genes that can be used for transformation of most filamentous fungi and yeasts such as acetamidase genes or cDNAs (the *amdS*, *niaD*, *facA* genes or cDNAs from *A.nidulans*, *A.oryzae*, or *A.niger*), or genes providing resistance to antibiotics like G418, hygromycin, bleomycin, kanamycin, phleomycin or benomyl resistance (*benA*). Alternatively, specific selection markers can be used such as auxotrophic markers which require corresponding mutant host strains: e.g. *URA3* (from *S.cerevisiae* or analogous genes from other yeasts), *pyrG* or *pyrA* (from *A.nidulans* or *A.niger*), *argB* (from *A.nidulans* or *A.niger*) or *trpC*. In a preferred embodiment the selection marker is deleted from the transformed host cell after introduction of the expression construct so as to obtain transformed host cells capable of producing the polypeptide which are free of selection marker genes.

Other markers include ATP synthetase subunit 9 (*oliC*), orotidine-5'-phosphate- decarboxylase (*pvrA*), the bacterial G418 resistance gene (useful in yeast, but not in filamentous fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli uidA* gene, coding for glucuronidase (GUS). Vectors may be used *in vitro*, for example for the production of RNA or to transfect or transform a host cell.

For most filamentous fungi and yeast, the expression construct is preferably integrated into the genome of the host cell in order to obtain stable transformants. However, for certain yeasts suitable episomal vector systems are also available into which the expression construct can be incorporated for stable and high level expression. Examples thereof include vectors derived from the 2 μ m, CEN and pKD1 plasmids of *Saccharomyces* and *Kluyveromyces*, respectively, or vectors containing an AMA sequence (e.g. AMA1 from *Aspergillus*). When expression constructs are integrated into host cell genomes, the constructs are either integrated at random loci in the genome, or at predetermined target loci using homologous

recombination, in which case the target loci preferably comprise a highly expressed gene. A highly expressed gene is a gene whose mRNA can make up at least 0.01% (w/w) of the total cellular mRNA, for example under induced conditions, or alternatively, a gene whose gene product can make up at least 0.2% (w/w) of the total cellular protein, or, in case of a secreted gene product, can be secreted to a level of at least 0.05 g/l.

An expression construct for a given host cell will usually contain the following elements operably linked to each other in consecutive order from the 5'-end to 3'-end relative to the coding strand of the sequence encoding the polypeptide of the first aspect: (1) a promoter sequence capable of directing transcription of the DNA sequence encoding the polypeptide in the given host cell, (2) preferably, a 5'-untranslated region (leader), (3) optionally, a signal sequence capable of directing secretion of the polypeptide from the given host cell into the culture medium, (4) the DNA sequence encoding a mature and preferably active form of the polypeptide, and preferably also (5) a transcription termination region (terminator) capable of terminating transcription downstream of the DNA sequence encoding the polypeptide.

Downstream of the DNA sequence encoding the polypeptide, the expression construct preferably contains a 3' untranslated region containing one or more transcription termination sites, also referred to as a terminator. The origin of the terminator is less critical. The terminator can for example be native to the DNA sequence encoding the polypeptide. However, preferably a yeast terminator is used in yeast host cells and a filamentous fungal terminator is used in filamentous fungal host cells. More preferably, the terminator is endogenous to the host cell in which the DNA sequence encoding the polypeptide is expressed.

Enhanced expression of the polynucleotide encoding the polypeptide of the invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, signal sequence and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the invention.

Aside from the promoter native to the gene encoding the polypeptide of the invention, other promoters may be used to direct expression of the polypeptide of the invention. The promoter may be selected for its efficiency in directing the expression of the polypeptide of the invention in the desired expression host.

Promoters/enhancers and other expression regulation signals may be selected to be compatible with the host cell for which the expression vector is

designed. For example prokaryotic promoters may be used, in particular those suitable for use in *E.coli* strains. When expression of the polypeptides of the invention is carried out in mammalian cells, mammalian promoters may be used. Tissues-specific promoters, for example hepatocyte cell-specific promoters, may also be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, herpes simplex virus promoters or adenovirus promoters.

Suitable yeast promoters include the *S. cerevisiae* GAL4 and ADH promoters and the *S. pombe* nmt1 and adh promoter. Mammalian promoters include the metallothionein promoter which can be induced in response to heavy metals such as cadmium. Viral promoters such as the SV40 large T antigen promoter or adenovirus promoters may also be used. All these promoters are readily available in the art.

Mammalian promoters, such as β -actin promoters, may be used. Tissue-specific promoters, in particular endothelial or neuronal cell specific promoters (for example the DDAH1 and DDAHII promoters), are especially preferred. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art.

A variety of promoters can be used that are capable of directing transcription in the host cells of the invention. Preferably the promoter sequence is derived from a highly expressed gene as previously defined. Examples of preferred highly expressed genes from which promoters are preferably derived and/or which are comprised in preferred predetermined target loci for integration of expression constructs, include but are not limited to genes encoding glycolytic enzymes such as triose-phosphate isomerases (TPI), glyceraldehyde-phosphate dehydrogenases (GAPDH), phosphoglycerate kinases (PGK), pyruvate kinases (PYK), alcohol dehydrogenases (ADH), as well as genes encoding amylases, glucoamylases, proteases, xylanases, cellobiohydrolases, β -galactosidases, alcohol (methanol) oxidases, elongation factors and ribosomal proteins. Specific examples of suitable highly expressed genes include e.g. the LAC4 gene from *Kluyveromyces* sp., the methanol oxidase genes (AOX and MOX) from *Hansenula* and *Pichia*, respectively, the glucoamylase (glaA) genes from *A.niger* and *A.awamori*, the *A.oryzae* TAKA-amylase gene, the *A.nidulans* gpdA gene and the *T.reesei* cellobiohydrolase genes.

Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (xlnA), phytase, ATP-synthetase subunit 9 (oliC), triose phosphate isomerase (tpi), alcohol dehydrogenase (AdhA), amylase (amy), amyloglucosidase (AG - from the glaA gene), acetamidase (amdS) and glyceraldehyde-3-phosphate dehydrogenase (gpd) promoters.

Examples of strong yeast promoters which may be used include those obtainable from the genes for alcohol dehydrogenase, lactase, 3-phosphoglycerate kinase and triosephosphate isomerase.

Examples of strong bacterial promoters which may be used include the amylase and SPo2 promoters as well as promoters from extracellular protease genes.

Promoters suitable for plant cells which may be used include napaline synthase (nos), octopine synthase (ocs), mannopine synthase (mas), ribulose small subunit (rubisco ssu), histone, rice actin, phaseolin, cauliflower mosaic virus (CMV) 35S and 19S and circovirus promoters.

The vector may further include sequences flanking the polynucleotide giving rise to RNA which comprise sequences homologous to ones from eukaryotic genomic sequences, preferably mammalian genomic sequences, or viral genomic sequences. This will allow the introduction of the polynucleotides of the invention into the genome of eukaryotic cells or viruses by homologous recombination. In particular, a plasmid vector comprising the expression cassette flanked by viral sequences can be used to prepare a viral vector suitable for delivering the polynucleotides of the invention to a mammalian cell. Other examples of suitable viral vectors include herpes simplex viral vectors and retroviruses, including lentiviruses, adenoviruses, adeno-associated viruses and HPV viruses (such as HPV-16 or HPV-18). Gene transfer techniques using these viruses are known to those skilled in the art. Retrovirus vectors for example may be used to stably integrate the polynucleotide giving rise to the antisense RNA into the host genome. Replication-defective adenovirus vectors by contrast remain episomal and therefore allow transient expression.

The vector may contain a polynucleotide of the invention oriented in an antisense direction to provide for the production of antisense RNA. This may be used to reduce, if desirable, the levels of expression of the polypeptide.

Host Cells and Expression

In a further aspect the invention provides a process for preparing a

polypeptide of the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions suitable for expression by the vector of a coding sequence encoding the polypeptide, and recovering the expressed polypeptide. Polynucleotides of the invention can be
5 incorporated into a recombinant replicable vector, such as an expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making a polynucleotide of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under
10 conditions which bring about the replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect cells such as Sf9 cells and (e.g. filamentous) fungal cells.

Preferably the polypeptide is produced as a secreted protein in which
15 case the DNA sequence encoding a mature form of the polypeptide in the expression construct is operably linked to a DNA sequence encoding a signal sequence. In the case where the gene encoding the secreted protein has in the wild type strain a signal sequence preferably the signal sequence used will be native (homologous) to the DNA sequence encoding the polypeptide. Alternatively the signal sequence is foreign
20 (heterologous) to the DNA sequence encoding the polypeptide, in which case the signal sequence is preferably endogenous to the host cell in which the DNA sequence is expressed. Examples of suitable signal sequences for yeast host cells are the signal sequences derived from yeast MFalpha genes. Similarly, a suitable signal sequence for filamentous fungal host cells is e.g. a signal sequence derived from a filamentous
25 fungal amyloglucosidase (AG) gene, e.g. the *A.niger* glaA gene. This signal sequence may be used in combination with the amyloglucosidase (also called (gluco)amylase) promoter itself, as well as in combination with other promoters. Hybrid signal sequences may also be used within the context of the present invention.

Preferred heterologous secretion leader sequences are those
30 originating from the fungal amyloglucosidase (AG) gene (glaA - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the MFalpha gene (yeasts e.g. *Saccharomyces* and *Kluyveromyces*) or the alpha-amylase gene (*Bacillus*).

The vectors may be transformed or transfected into a suitable host cell as described above to provide for expression of a polypeptide of the invention.
35 This process may comprise culturing a host cell transformed with an expression vector as described above under conditions suitable for expression of the polypeptide, and

optionally recovering the expressed polypeptide.

A further aspect of the invention thus provides host cells transformed or transfected with or comprising a polynucleotide or vector of the invention. Preferably the polynucleotide is carried in a vector which allows the replication and expression of
5 the polynucleotide. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), or eukaryotic fungal, yeast or plant cells.

The invention encompasses processes for the production of a polypeptide of the invention by means of recombinant expression of a DNA sequence
10 encoding the polypeptide. For this purpose the DNA sequence of the invention can be used for gene amplification and/or exchange of expression signals, such as promoters, secretion signal sequences, in order to allow economic production of the polypeptide in a suitable homologous or heterologous host cell. A homologous host cell is herein defined as a host cell which is of the same species or which is a variant within the
15 same species as the species from which the DNA sequence is derived.

Suitable host cells are preferably prokaryotic microorganisms such as bacteria, or more preferably eukaryotic organisms, for example fungi, such as yeasts or filamentous fungi, or plant cells. In general, yeast cells are preferred over filamentous fungal cells because they are easier to manipulate. However, some proteins are either
20 poorly secreted from yeasts, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a filamentous fungal host organism should be selected.

Bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other
25 bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*. A preferred yeast host cell for the expression of the DNA sequence encoding the polypeptide is one of the genus *Saccharomyces*, *Kluyveromyces*, *Hansenula*, *Pichia*, *Yarrowia*, or *Schizosaccharomyces*. More preferably, a yeast host cell is selected from the group consisting of the species *Saccharomyces cerevisiae*, *Kluyveromyces lactis*
30 (also known as *Kluyveromyces marxianus* var. *lactis*), *Hansenula polymorpha*, *Pichia pastoris*, *Yarrowia lipolytica*, and *Schizosaccharomyces pombe*.

Most preferred for the expression of the DNA sequence encoding the polypeptide are, however, filamentous fungal host cells. Preferred filamentous fungal host cells are selected from the group consisting of the genera *Aspergillus*,
35 *Trichoderma*, *Fusarium*, *Disporotrichum*, *Penicillium*, *Acremonium*, *Neurospora*, *Thermoascus*, *Myceliophthora*, *Sporotrichum*, *Thielavia*, and *Talaromyces*. More

preferably a filamentous fungal host cell is of the species *Aspergillus oysae*, *Aspergillus sojae* or *Aspergillus nidulans* or is of a species from the *Aspergillus niger* Group (as defined by Raper and Fennell, The Genus *Aspergillus*, The Williams & Wilkins Company, Baltimore, pp 293-344, 1965). These include but are not limited to

5 *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus tubigenis*, *Aspergillus aculeatus*, *Aspergillus foetidus*, *Aspergillus nidulans*, *Aspergillus japonicus*, *Aspergillus oryzae* and *Aspergillus ficuum*, and also those of the species *Trichoderma reesei*, *Fusarium graminearum*, *Penicillium chrysogenum*, *Acremonium alabamense*, *Neurospora crassa*, *Myceliophthora thermophilum*, *Sporotrichum cellulophilum*, *Disporotrichum*

10 *dimorphosporum* and *Thielavia terrestris*.

Examples of preferred expression hosts within the scope of the present invention are fungi such as *Aspergillus* species (in particular those described in EP-A-184,438 and EP-A-284,603) and *Trichoderma* species; bacteria such as *Bacillus* species (in particular those described in EP-A-134,048 and EP-A-253,455), especially

15 *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Pseudomonas* species; and yeasts such as *Kluyveromyces* species (in particular those described in EP-A-096,430 such as *Kluyveromyces lactis* and in EP-A-301,670) and *Saccharomyces* species, such as *Saccharomyces cerevisiae*.

Host cells according to the invention include plant cells, and the

20 invention therefore extends to transgenic organisms, such as plants and parts thereof, which contain one or more cells of the invention. The cells may heterologously express the polypeptide of the invention or may heterologously contain one or more of the polynucleotides of the invention. The transgenic (or genetically modified) plant may therefore have inserted (typically stably) into its genome a sequence encoding the

25 polypeptides of the invention. The transformation of plant cells can be performed using known techniques, for example using a Ti or a Ri plasmid from *Agrobacterium tumefaciens*. The plasmid (or vector) may thus contain sequences necessary to infect a plant, and derivatives of the Ti and/or Ri plasmids may be employed.

The host cell may overexpress the polypeptide, and techniques for

30 engineering over-expression are well known and can be used in the present invention. The host may thus have two or more copies of the polynucleotide.

Alternatively, direct infection of a part of a plant, such as a leaf, root or stem can be effected. In this technique the plant to be infected can be wounded, for example by cutting the plant with a razor, puncturing the plant with a needle or rubbing

35 the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The plant or plant part can then be grown on a suitable culture medium and allowed to

develop into a mature plant. Regeneration of transformed cells into genetically modified plants can be achieved by using known techniques, for example by selecting transformed shoots using an antibiotic and by sub-culturing the shoots on a medium containing the appropriate nutrients, plant hormones and the like.

5

Culture of host cells and recombinant production

The invention also includes cells that have been modified to express the proline specific endoprotease or a variant thereof. Such cells include transient, or preferably stably modified higher eukaryotic cell lines, such as mammalian cells or
10 insect cells, lower eukaryotic cells, such as yeast and filamentous fungal cells or prokaryotic cells such as bacterial cells.

It is also possible for the polypeptides of the invention to be transiently expressed in a cell line or on a membrane, such as for example in a baculovirus expression system. Such systems, which are adapted to express the
15 proteins according to the invention, are also included within the scope of the present invention.

According to the present invention, the production of the polypeptide of the invention can be effected by the culturing of microbial expression hosts, which have been transformed with one or more polynucleotides of the present invention, in a
20 conventional nutrient fermentation medium.

The recombinant host cells according to the invention may be cultured using procedures known in the art. For each combination of a promoter and a host cell, culture conditions are available which are conducive to the expression the DNA sequence encoding the polypeptide. After reaching the desired cell density or
25 titre of the polypeptide the culturing is ceased and the polypeptide is recovered using known procedures.

The fermentation medium can comprise a known culture medium containing a carbon source (e.g. glucose, maltose, molasses, etc.), a nitrogen source (e.g. ammonium sulphate, ammonium nitrate, ammonium chloride, etc.), an organic
30 nitrogen source (e.g. yeast extract, malt extract, peptone, etc.) and inorganic nutrient sources (e.g. phosphate, magnesium, potassium, zinc, iron, etc.). Optionally, an inducer (dependent on the expression construct used) may be included or subsequently be added.

The selection of the appropriate medium may be based on the choice
35 of expression host and/or based on the regulatory requirements of the expression construct. Suitable media are well-known to those skilled in the art. The medium may, if

desired, contain additional components favoring the transformed expression hosts over other potentially contaminating microorganisms.

The fermentation may be performed over a period of from 0.5-30 days. Fermentation may be a batch, continuous or fed-batch process, at a suitable
5 temperature in the range of between 0°C and 45°C and, for example, at a pH from 2 to 10. Preferred fermentation conditions include a temperature in the range of between 20°C and 37°C and/or a pH between 3 and 9. The appropriate conditions are usually selected based on the choice of the expression host and the protein to be expressed.

After fermentation, if necessary, the cells can be removed from the
10 fermentation broth by means of centrifugation or filtration. After fermentation has stopped or after removal of the cells, the polypeptide of the invention may then be recovered and, if desired, purified and isolated by conventional means. The proline specific endoprotease of the invention can be purified from fungal mycelium or from the culture broth into which the proline specific endoprotease is released by the cultured
15 fungal cells.

In a preferred embodiment the polypeptide is obtained from a fungus, more preferably from an *Aspergillus*, most preferably from *Aspergillus niger*.

Modifications

20 Polypeptides of the invention may be chemically modified, e.g. post-translationally modified. For example, they may be glycosylated (one or more times) or comprise modified amino acid residues. They may also be modified by the addition of histidine residues to assist their purification or by the addition of a signal sequence to promote secretion from the cell. The polypeptide may have amino- or carboxyl-
25 terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain.

A polypeptide of the invention may be labelled with a revealing label. The revealing label may be any suitable label which allows the polypeptide to be
30 detected. Suitable labels include radioisotopes, e.g. ¹²⁵I, ³⁵S, enzymes, antibodies, polynucleotides and linkers such as biotin.

The polypeptides may be modified to include non-naturally occurring amino acids or to increase the stability of the polypeptide. When the proteins or peptides are produced by synthetic means, such amino acids may be introduced during
35 production. The proteins or peptides may also be modified following either synthetic or recombinant production.

The polypeptides of the invention may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the C to N orientation. This is conventional in the art for producing such proteins or peptides.

5 A number of side chain modifications are known in the art and may be made to the side chains of the proteins or peptides of the present invention. Such modifications include, for example, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with NaBH_4 , amidination with methylacetimidate or acylation with acetic anhydride.

10 The sequences provided by the present invention may also be used as starting materials for the construction of "second generation" enzymes. "Second generation" proline specific proteases are proline specific proteases, altered by mutagenesis techniques (e.g. site-directed mutagenesis), which have properties that differ from those of wild-type proline specific protease or recombinant proline specific proteases such as those produced by the present invention. For example, their
15 temperature or pH optimum, specific activity, substrate affinity or thermostability may be altered so as to be better suited for use in a particular process.

Amino acids essential to the activity of the proline specific endoprotease of the invention, and therefore preferably subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis
20 or alanine-scanning mutagenesis. In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g. proline specific endoprotease activity) to identify amino acid residues that are critical to the activity of the molecule. Sites of enzyme-substrate interaction can also be determined by analysis of crystal structure as determined by
25 such techniques as nuclear magnetic resonance, crystallography or photo-affinity labelling.

The use of yeast and filamentous fungal host cells is expected to provide for such post-translational modifications (e.g. proteolytic processing, myristilation, glycosylation, truncation, and tyrosine, serine or threonine
30 phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the invention.

Preparations

35 Polypeptides of the invention may be in an isolated form. It will be understood that the polypeptide may be mixed with carriers or diluents which will not interfere with the intended purpose of the polypeptide and still be regarded as isolated.

A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the polypeptide in a preparation in which more than 70%, e.g. more than 80%, 90%, 95%, 98% or 99% of the proteins in the preparation is a polypeptide of the invention.

5 Polypeptides of the invention may be provided in a form such that they are outside their natural cellular environment. Thus, they may be substantially isolated or purified, as discussed above, or in a cell in which they do not occur in nature, for example a cell of other fungal species, animals, plants or bacteria.

10 The invention also relates to the use of a prolyl-specific endoprotease in the preparation of a beverage. A prolyl-specific endoprotease is used preferably in the preparation of beer, wine or fruit juice. By the addition of a prolyl-specific endoprotease according to the method according to the invention, a reduction or prevention of haze is achieved. By adding these prolyl-specific endoproteases, new beverages are obtained. Thus, the invention also relates to beverages obtainable by the method according to the invention. These beverages include for example beer, wine and fruit juice obtainable by a method according to the invention.

15 An advantage of the beverages obtainable by the method according to the invention is that these beverages have a high content of anti-oxidants. Polyphenols are anti-oxidants. Beer is usually treated with a polyphenol-removing agent to prevent the formation of haze, and as a result the beer obtained has a low antioxidant activity. The same is true for other beverages treated with poly-phenol removing agents. Beer obtainable by the method according to the invention has a higher endogenous anti-oxidant activity. Because anti-oxidants are seen as health improving ingredients, the beverages obtainable by the method according to the invention may be considered as beverages that are healthier than the same type of beverage prepared with polyphenol removing agents, such as PVPP. It is an advantage of the method according to the invention that the color of fruit juices obtainable by the method is not or less faded than the color of fruit juices obtained after the removal of polyphenols. Wines and fruit juices obtainable by the method according to the invention have improved aroma and flavour in comparison to beverages obtained by a method wherein bentonite or a similar compound is used, since bentonite not only removes proteins but also aroma and/or flavour components.

35 **EXAMPLES**

EXAMPLE 1

Materials

Proline-specific endoprotease enzymes (Endo-Pro's)

5 *Aspergillus niger* G306 was deposited with the CBS (CBS109712) on
10 September 2001. A niger G306 contains a gene encoding a prolyl-specific
endoprotease according to the invention. The gene or cDNA obtainable from this
organism may be cloned and expressed in any *Aspergillus niger* host using known
methods.

10

The following samples were used:

1) "Endo-Pro A", a proline-specific endoprotease was used in experiments with beer.

The sample was an ultrafiltration concentrate obtained after ultrafiltration of a
fermentation broth obtained after fermentation of an *Aspergillus niger* strain comprising
15 a gene coding for a proline-specific endoprotease. The prolyl-specific activity of the
Endo-pro A sample was 5.06 U/ml, determined as described under Methods. The
protein concentration was estimated to be 50g/l, based on the specific activity of a
sample of prolyl-specific endoprotease with a purity higher than 90%.

2) "Endo-Pro B", an proline-specific endoprotease was used in experiments with wine.

20 The sample was obtained after purification over a column and had an activity of
6.0 U/ml.

Papain

25 Collupulin, a liquid papain preparation available from DSM, France
was used for experiments with papain. The activity is 5280 NF/mg. One unit NF is the
quantity of papain activity that catalyzes the hydrolysis of casein to produce one
microgramme equivalent of soluble tyrosine per hour at pH 6.0. The protein
concentration in the papain sample was measured, which is 119 g/l (Lowry).

30 Polyvinylpolypyrrolidone (PVPP)

PVPP used was a commercially available non-water soluble PVPP
under the name 'Polyclar AT'.

Beer

35 A malt beer (pilsener type) from "Les Trois Brasseurs" in Lille,
France, was used in all experiment performed with beer. The alcohol percentage of

this beer was 5.2% (v/v) and the pH was 4.4. This particular beer was chosen because of the relative high amount of turbidity measured in this beer upon chilling, in comparison with other commercially available beers. The beer had a protein concentration of 0.9 g/l, as determined by Lowry's-method.

5

White wine

A white wine prepared from white Sauvignon grapes was used without any protein removal treatment. The alcoholic fermentation during wine preparation was performed with a selected yeast VL3 from Lallemend. The oenologic analysis of the wine gave the following results:

10

Sugars (g/l)	1.1
Ethanol %vol	12.97
Total Acidity (g H ₂ SO ₄ /l)	4.14
Volatil acidity (g H ₂ SO ₄ /l)	0.22
PH	3.46
Free SO ₂ (mg/l)	18
Total SO ₂ (mg/l)	96
Glycerol (g/l)	3
Tartric acid (g/l)	3
Malic acid (g/l)	2.8
Lactic acid (g/l)	0.1
Level of Folin	7

Example 2

15

Methods

Spectrophotometric method for determining enzyme activities

To measure the activity of the proline-specific endoprotease, a substrate solution of 2 mM solution of N-carbobenzoxy-glycine-proline-p-nitro anilide (Z-Gly-Pro-pNA; m.w. 426.43 ; Bachem, Switzerland) made in a 0.1 M citric acid / 0.2 M disodium phosphate buffer pH 5.0 containing 40 % dioxan is used.

20

To 1 mL of buffer pH 5.0, 250 µl of the substrate solution is added followed by 100 µl of the enzyme solution (larger or smaller volume amounts of enzyme solution should be

compensated for by buffer solution). The reaction mixture is incubated at 37°C and the release of pNA is followed by measuring the absorbance increase at 410 nm.

One unit is defined as the enzyme activity that liberates 1µmol pNA from Z-Gly-Pro-pNA in 1 minute under described reaction conditions using a molar extinction coefficient (E) of 8,800 M⁻¹.

The activity of the glycine-specific endoprotease isolated from Flavourzyme 1000 L (NOVO, Denmark) was measured on the synthetic chromogenic substrate Z-Gly-Gly-pNA at pH 5.0 and 37 degrees C using procedures as described. One unit of glycine-specific endoprotease is defined as the quantity of enzyme that provokes the release of 1 micromol of pNA from Z-Gly-Gly-pNA per minute at pH 5.0 and 37 degrees C.

The activity of the proline-specific carboxypeptidase from *Xanthomonas* species was measured by measuring the quantity of proline residues released from the synthetic peptide Z-Pro-Pro (Bachem, Switzerland) using an amino acid analyzer. One unit is the quality of enzyme that provokes the release of 1 micromol of proline from Z-Pro-Pro per hour at pH 7.0 and 37 degrees C.

Chill Haze measurements (Alcohol/low-temperature test according to Lucien Chapon)

Turbidity or haze was measured with a turbidimeter ("Tannometer", Pfeuffer Gmbh, Kitzingen, Germany) in line with the operating instructions. In cold beer a reversible turbidity can occur which is caused by precipitated polyphenol-protein complexes. The addition of extra alcohol accelerates the formation of these turbidities. In the "chill haze test" this phenomenon is used to quantitate the polyphenol-protein complexes present. To calibrate the Tannometer, a formazine standard solution was prepared as described (Jean de Clerk, "Cours de Brasseries" 2nd Edition, Vol 2, 1963, pp.595-596, Université de Louvain, Belgium). The beer haze or turbidity unit used is the ECB which is nephelometric turbidity units as recommended by the European Brewery Convention. Chill haze tests as described for beer may also be performed with alcohol free beers or worts (adapted from the Operating Instruction Guide of the Tannometer). In these cases ethanol is also added to the samples in an amount sufficient to reach an alcohol content of 10% (v/v) in the samples. Ethanol was added to all wort samples to reach 10% (v/v) after which each sample is cooled to -8°C during 30 min. Then the haze formed (Turbidity, in units EBC) is quickly measured in the turbidimeter with its measuring chamber also at -8°C.

Heating haze tests

As described by K.J. Siebert (K.J. Siebert et al, J. Agric. Food Chem. 44 (1996)) haze in beverages like wine or fruit juices can be induced by a heating test. The amount of haze formed is mainly a function of the levels of haze-active proteins and polyphenols in the beverage. In the Heating test, the turbidity of samples (of for example wine or fruit juice) is measured with a turbidimeter before and after heating at 80°C during 30 min. Before measuring the turbidity, the heated sample is cooled down under cold water until a temperature of 22-25 °C is reached. In the wine trials (see example III) the calibration of the turbidimeter was performed with NTU-formazine standard solutions, for the fruit juice trials (see example V) the NTU turbidity standard solution was purchased from Reagecon, Ireland. NTU= nephelometric turbidity units.

Control experiments

- (i) A blank experiment was performed wherein no exogenous protein was added during the incubation.
- (ii) An experiment was carried out wherein beer was used that had been treated with a large amount of PVPP (1000 g/hl) before incubation. This experiment allowed the determination of the average amount of haze induced by the chill haze test which is due to polyphenol-protein precipitate, because PVPP removes polyphenols from the beer, and thus interferes with formation of haze.
- (iii) Experiments were performed wherein exogenous proteins (prolyl-specific endoprotease or papain, respectively) were added to beer cooled to 0°C after incubation at 40°C for 1 hour. Incubation at 0°C took place for 15 minutes prior to haze measurements. Since the enzyme and papain are not or hardly active at 0°C, these experiments allowed discrimination between the enzyme activity effect and non-enzymatic protein effects.

Example 3

Effects of the addition of a prolyl-specific endoprotease on haze formation in beer

To a decarbonated malt beer (Les Trois Brasseurs), protein content: 0.9 g/l, various amounts of a prolyl-specific endoprotease enzyme ("Endo-Pro A", see Materials) were added. Two series of haze-measurements were performed. In the first series, the beer-Endo-Pro A compositions were incubated at 40°C for 1 hour prior to the Chill haze test. After incubation at 40°C, and just prior to the Chill haze test, ethanol was added to the beer-Endo-Pro A composition in an amount sufficient to

increase the alcohol content to 6% (v/v). In the second series, the beer without Endo-Pro A was incubated at 40°C for 1 hour, and then cooled to 0°C. At 0°C, the Endo-Pro A was added and the resulting compositions were incubated at 0°C for 15 min. Just prior to the Chill haze test, ethanol was added to the beer-Endo-Pro A composition in an amount sufficient to increase the alcohol content to 6% (v/v).

The amounts of Endo-Pro A added and the percentage of haze reduction relative to the haze measured when no Endo-Pro A was added are shown in Table 1. The amounts of Endo-Pro A added covered a large range from less than 1% of exogenous proteins added to more than 10 %, relative to the amount of protein present in the beer.

Table 1. Effect of the addition of a prolyl-specific endoprotease enzyme to a beer on the amount of haze after incubation at 40°C for 1 h

Trial	"Endo-Pro A" added		Chill Haze test (EBC)	Haze reduction (%)
	µl/10ml of beer	% of exogenous protein added*		
1	0	0	141	0
2	0.45	0.25	116	17.7
3	0.9	0.5	101	28.4
4	1.8	1	87.4	38.0
5	3.6	2	81.1	42.5
6	5.4	3	69.0	51.1
7	9	5	61.3	56.5
8	18	10	48.5	65.6
9	36	20	39.9	71.7
10	54	30	33.4	76.3

* "% of exogenous enzyme added" reflects the amount of Endo-Pro A"-enzyme added expressed as a percentage of the total amount of proteins present in the beer before addition of the enzyme.

Table 2. Effect of prolyl-specific endoprotease enzyme to a beer on the amount of haze after incubation at 0°C for 15 min

Trial	Endo-Pro added		Chill Haze test (EBC)	Haze reduction (%)
	µl/10ml of beer	% of exogenous protein added		
1	0	0	141	0
2	0.45	0.25	141	0
3	0.9	0.5	141	0
4	1.8	1	143	-1.4
5	3.6	2	145	-2.8
6	5.4	3	138	2.1
7	9	5	138	2.1
8	18	10	130	7.8
9	36	20	126	10.6
10	54	30	120	14.9

Table 1 clearly illustrates that less haze is formed upon chilling when a prolyl-specific endoprotease is added to beer at a temperature when the protease is active prior to chilling. Table 2 clearly illustrates that there is some effect when a prolyl-specific endoprotease is added to the beer at a temperature so low that the protease is not or hardly active, but the effect is very small in comparison to the effect observed when the protease is added at a temperature where it is active.

10 Example 4

Effects of the addition of Papain on haze formation in beer

To a decarbonated malt beer (Les Trois Brasseur), protein content: 0.9 g/l, various amounts of papain (from 0 to 100 g/hl) were added. Two series of chill haze-measurements were performed. For the first series, the beer-papain compositions were incubated at 40°C for 1 hour prior to the Chill haze test. Ethanol was added to the incubated samples to reach 6% alcohol (v/v) prior to the haze measurements. In the second series, beer samples were incubated at 40°C for 1 hour and subsequently cooled to 0°C. Then, papain was added and the samples were incubated at 0°C for 15 min. The amounts of papain added and the percentage of haze reduction relative to the haze measured when no papain was added are shown in Table 3.

Table 3. Effect of papain on the amount of haze formed in beer after incubation at 40°C for 1 h

Trial	Papain added		Chill Haze test (EBC)	Haze reduction (%) (%)
	g/hl beer	% of exogeneous protein added		
1	0	0	143	0
2	0.2	0.03	140	2.1
3	0.5	0.07	133	7.0
4	1	0.13	119	16.8
5	2	0.26	94.1	34.2
6	3 ⁽¹⁾	0.40	91.6	35.9
7	5	0.66	83.8	41.4
8	8	1.06	82.4	42.4
9	10	1.32	84.0	41.3
10	100	13.22	81.6	42.9

(1) 3 g/hl is the maximal dosage recommended

5 Table 4. Effect of papain on the amount of haze after incubation at 0°C for 15 min

Trial	Papain added		Chill Haze test (EBC)C	Haze reduction (%)
	g/hl beer	% of exogeneous protein added		
1	0	0	143	0
2	0.2	0.03	139	2.8
3	0.5	0.07	136	4.9
4	1	0.13	135	5.6
5	2	0.26	134	6.3
6	3	0.40	132	7.7
7	5	0.66	136	4.9
8	8	1.06	130	9.1
9	10	1.32	122	14.7
10	100	13.22	138	3.5

The results in Table 3 illustrate the effect of papain on the amount of haze formed in beer upon chilling. It is clear that the effect of papain on haze levels off when papain is added in an amount of 3 g/hl and higher. Apparently, it is not possible to achieve the

same amount of haze reduction with papain as with a prolyl-specific endoprotease.

Example 5

5 Effects of the addition of PVPP on haze formation in beer

In both beer-prolyl-specific endoprotease experiments and the beer-papain experiments, a control experiment was done by adding a large amount of PVPP (1000 g/hl) to the beer prior to incubation. After 15 min of mixing the PVPP was removed by filtration (No prolyl-specific endoprotease enzyme or papain was added). In both controls almost no haze were formed during the chill haze test. Since it is known that PVPP removes polyphenols from beverages, these control experiments indicate that polyphenols do take part in haze formation in beer. To measure the PVPP effect on beer haze stability, different amounts of PVPP were added to a decarbonated beer and removed by filtration after 15 min of mixing. Prior to adding the PVPP the beer was incubated during 1h at 40°C.

Table 5 shows the effect of the addition of various amounts of PVPP on the amount of haze present in beer upon chilling. No Endo-Pro A or papain was added.

Table 5. Effect of PVPP on the amount of haze present in beer upon chilling

PVPP added (g/hl)	Chill Haze test (EBC)	Haze reduction (%)
0	133	0
10	131	1.5
20	115	13.5
30 ⁽¹⁾	103	22.6
50	65.2	51.0
80	48.4	63.6
100	42.1	68.3
500	17.2	87.1
1000	9.5	92.9

(1) 30g/hl maximal dosage used in breweries

In Table 3 it is shown that the addition of 3g of Papain / hl of beer (which is the maximal dosage recommended in the beer industry) after incubation at 40°C for 1 hour induces an haze decrease of almost 36%. In the case of addition of the prolyl-specific endoprotease, the addition of 1% (relative to the amount of protein in the

beer) of prolyl-specific endoprotease after incubation at 40°C for 1 hour induces a decrease of a beer chill haze of 38% (see Table 1). In breweries, PVPP is generally added in a quantity that does not exceed 30 g/hl. Since PVPP reduces haze by around 20% when it is added in that quantity, it can be concluded that both papain and prolyl-specific endoprotease enzymes are better haze inhibitors than PVPP.

Example 6

Endo-Pro addition on a 100% malt mash and haze reduction in a 100% malt wort

The objective was to determine if the addition of endo-proA to a 100% malt mash could result in an haze reduction in the final malt wort.

Each mashing trial begins with the mixing of 25 g of milled malt with 100 ml of water. Then, the mash is heated to 50°C and after the addition of an amount of "Endo-Pro A" the mash is treated according to a step-wise heating procedure to four successively higher temperatures. Table 6 shows that mashing temperature profile. During all the mashing the mash was stirred at 200 rpm. At the end of the mashing, the mash is kept at room temperature and water was added to compensate the water evaporation. Then, the mash was filtered on paper to separate the wort (liquid) from the solids.

Table 6. Mashing temperature profile

Steps	Temperature	time
1	50°C	30 min
temperature increase	1°C/min	13 min
2	63°C	30 min
temperature increase	1°C/min	10 min
3	72°C	30 min
temperature increase	1°C/min	4 min
4	77°C	10 min

0, 200 & 500 µl Endo-Pro A was added to the mash, respectively. A control experiment was performed wherein 500µl of Endo-Pro A was used that was deactivated by heating it to 90°C during 15 min. The turbidity or haze of the wort was measured at room temperature and after a chill haze test. Wort chill tests are performed as described in the Alcohol/low-temperature test according to Chapon (Chill

Haze test –Pfeuffer Operating instructions) adding ethanol to reach 10% (v/v) in the sample as recommended by Chapon for alcohol-free beers.

5 Table 7: effect of the addition of prolyl-specific endoprotease to 100% malt mashes on the amount of haze formed in the resulting 100% malt worts (after Chill haze test)

Trial	Endo-Pro A added in the mash (µl)	Wort initial turbidity (EBC ⁽¹⁾)	Wort turbidity after Chill Haze test (EBC)	Haze induced by the Chill Haze test (ΔEBC)	Endo-Pro A haze reduction effect (%)
1	0	10	158.5	148.5	0
2	200	15.9	59.2	43.3	70.8
3	500	22.5	49.1	26.6	82.1
4	500 (desactivated)	12.1	160.5	148.4	0.1

(1) EBC: nephelometric turbidity units recommended by the European Brewery Convention

10 The results in Table 7 clearly indicate that when a prolyl-specific endoprotease has been added to a malt mash, the resulting wort is much less turbid upon cooling than a wort prepared without the addition of a prolyl-specific endoprotease.

15 To study the Endo-Pro A effect, a chill haze test was performed on malt wort. It was observed that the addition of a prolyl-specific endoprotease decreased the wort chill haze. A decrease in haze formed in the Chill haze test was observed even at low quantities of prolyl-specific endoprotease enzyme added. When the enzyme is deactivated prior to be added in the mash, the stabilization effect disappears completely i.e. the amount of haze formed is no longer reduced. The haze decrease induced by the addition of a prolyl-specific enzyme is very important. In the example a haze reduction of up to 82 % was achieved.

20 In order to compare the effects of the addition of a prolyl-specific endoprotease enzyme in malt worts and in barley worts, experiments were performed wherein different amounts of Endo-Pro A were added in barley

mashes. The pH were respectively 5.6 in malt worts and 6.1 in barley worts. The results obtained with barley worts chill haze tests showed that as observed previously for malt worts, the treatment of barley mashes by a prolyl-specific endoprotease induces an important reduction of barley worts chill haze. Both malt and barley mashes
5 treated with a prolyl-specific endoprotease result in highly stabilized worts but the effect is stronger in malt worts than in barley worts. Indeed, the addition in the mash of 200µl of Endo-Pro A induced a haze reduction of around 59% in barley worts and more than 70% in malt worts. The trials performed with 500µl of Endo-Pro A increased the haze reduction until 82% in malt worts and did not improve the haze reduction in barley
10 worts compared to the 200µl Endo-Pro A experiment. Surprisingly, the addition of Endo-Pro A to barley mashes resulted in clear filtered worts while the non-Endo-Pro treated mashes resulted in cloudy filtered worts (the barley mash filtrations were performed at room temperature and the turbidity of the worts was measured at room temperature without ethanol addition). That effect is observed whatever the amount of
15 prolyl-specific endoprotease added in the barley mashes.

Example 7

Haze reduction in wine

Different dosages (0, 30, 60 150 µl) of a prolyl-specific endoprotease (Endo-Pro B) having a specific activity of 6.0 U/ml were added to flasks containing 500 ml of white wine (wine as described under "Materials") and incubated at room
20 temperature (22-25°C) for 19 days under a nitrogen atmosphere. The wine haze stability was measured after 0, 6, 8, 12 and 19 days using the Heating test as described under "Methods".
25

The results of the experiments are shown in Table 8. In Table 8, wine turbidity or haze is expressed in nephelos turbidity units (NTU), Δ NTU = turbidity in
30 NTU measured on wine samples after heating - turbidity in NTU measured on wine samples before heating. The quantity of bentonite required to stabilize the proteins of the wine was calculated according to the formula: $(1.48 \times \Delta \text{ NTU}) + 2$. As is known, less bentonite is needed to prevent haze formation in wine when a wine is less susceptible to haze formation.

Table 8. Effect of the addition of a prolyl-specific endoprotease enzyme to a white wine the amount of haze formed after heating the wine

Incubation time	Endo-Pro added (μ l/500ml)	Turbidity before heating (NTU)	Turbidity after heating (NTU)	Δ NTU	quantity of bentonite required (g/hl)	haze reduction (%)	Decrease of the quantity of bentonite required (%)
0	0 μ l (Control)	36.8	74.5	38	58		
6 days	0 μ l (Control)	25.8	63.8	38	58	0	0
	30 μ l	20.2	53.5	33	51	13	12
	60 μ l	21.6	56	34	53	11	9
	150 μ l	20.5	43.3	23	37	39	36
8 days	0 μ l (Control)	26.4	68	42	64	0	0
	30 μ l	31.4	60.2	29	45	31	30
	60 μ l	29.6	56.2	27	41	36	36
	150 μ l	25.1	48.4	23	36	45	44
12 days	0 μ l (Control)	24.4	50.1	26	40	0	0
	30 μ l	23	36.9	14	23	46	43
	60 μ l	23	36.6	14	23	46	43
	150 μ l	23.9	37.9	14	23	46	43
19 days	0 μ l (Control)	6.5	23.2	17	26	0	0
	30 μ l	5.8	11.2	5	10	71	62
	60 μ l	7	11.1	4	8	76	69
	150 μ l	8.5	13.7	5	10	71	62

endoprotease to a white wine before heating reduces the haze formed in the wine after heating. After 6 days of incubation at room temperature the effect is observed. Indeed, the haze decrease reaches 39% with 150 µl of the Endo-Pro B- added and around 12% with 30 µl or 60 µl of Endo-Pro B. After 12 days and whatever the amount of the prolyl-specific endoprotease used the haze reduction reaches 46% and exceeds 70% after 19 days. Therefore, it is clear that a prolyl-specific endoprotease can be used to avoid or to strongly reduce the quantity of bentonite required to stabilize wine against haze formation.

10 Example 8

Haze reduction in Strawberry Juice

A strawberry fruit juice was prepared as follows: strawberries were defrosted and crushed, and subsequently blanched (heated) at 90°C in order to destroy all endogenous enzymes such as polyphenol oxidases, and to denature proteins. The crushed strawberries were then cooled to 50°C, macerated for 30 min at 50°C with 600g/t of Rapidase BE super (a commercial enzyme product of DSM, France) and pressed in a pneumatic press. In order to remove the denatured proteins the resulting mixture was centrifuged at a speed of 8000 rpm and filtered. The strawberry-juice was collected. An acidified alcohol test was negative, which confirmed that the juice was pectin free. The pH value of the juice was 3.3.

Endo-Pro A/ strawberry juice incubations: Different volumes (0, 5, 10, 20 µl) of Endo-Pro A (5.06 U/ml) were added to 100 ml of strawberry juice and incubated at 50°C for 60 min. Two control experiments were performed, (i) one by adding 20µl of deactivated Endo-Pro A (incubated 30 min at 80°C) and (ii) a second control by adding 200 mg of PVPP to 20 ml of strawberry juice previously incubated 1h at 50°C. After having been mixed for 15 min at room temperature the PVPP was removed by centrifugation.

Juice Heating test: the juice turbidity was measured before and after heating the fruit juice samples at 80°C during 30 minutes. Before measuring the turbidity, the heated juices were cooled down under cold water.

The turbidity measurements were performed in a turbidimeter previously calibrated with NTU turbidity standards from Reagecon (Ireland)

Table 9: Effect of the addition of prolyl-specific endoprotease on the haze in strawberry juice

Trial	Endo-Pro A added (μ l/100ml)	Turbidity before heating (NTU)	Turbidity after heating (NTU)	Δ NTU	reduction effect (%)
1	0	10.1	17.5	7.4	0
2	5	9.5	15.0	5.5	25.7
3	10	9.4	15.4	6.0	18.9
5	10 (deactivated)	10.2	16.9	6.7	9.4
6	PVPP	1.4	2.3	0.9	

The results in Table 9 show that the addition of 5 μ l of Endo pro A in 100ml strawberry juice decreased haze formed after a juice heating test by 25.7%. The addition of 10 μ l of Endo-Pro A to 100ml strawberry juice did not improve the haze reduction effect compared to the 5 μ l trial. Possibly, the enzyme action is maximal with 5 μ l and with more enzyme addition more protein precipitation is obtained.

Deactivated enzyme still reduced the amount of haze formed, however, the effect was much less pronounced. After the addition of PVPP, hardly any haze was observed, but the color of the sample was also removed. The fact that the addition of PVPP results prevents haze formation indicates that also in strawberry juice haze is probably the result of polyphenol-protein interactions.

Example 9

15 Isolation of a proline-specific carboxypeptidase from *Xanthomonas* species

Although many scientific reports describe exopeptidases that are able to act on proline residues at the carboxyterminal ends of peptide chains, the hydrolysis rate of these carboxypeptidases towards proline residues is very low. Moreover most of these carboxypeptidases are not capable of releasing carboxyterminal proline residues from polyproline sequences. US 5,693,503 describes the purification of carboxypeptidases from *Xanthomonas* species that exhibit a surprisingly high activity towards the release of carboxyterminal proline residues, even from polyproline sequences. We have isolated such a carboxypeptidase from several *Xanthomonas* species to test their activity in preventing haze formation.

The isolation of the desired carboxypeptidase from *Xanthomonas*

proved to be a difficult task since the purification method as described in US 5,693,503 was not successful in our hands and a completely new purification protocol had to be developed. Of the many procedures tested the following protocol yielded substantially purified enzyme.

5 Starting from one liter of broth, cells were harvested by centrifugation, washed once with water and then disrupted by an ultrasonic treatment at 0 degrees C. Cell debris was removed by a centrifugation at 20000 rpm for 20 minutes and the resulting supernatant was recentrifuged under the same conditions. Then ammonium sulfate was added to the supernatant to reach 40% saturation. After low speed
10 centrifugation the supernatant was collected and additional ammonium sulfate was added to reach 80% saturation. The newly formed precipitate was collected by low speed centrifugation, extensively dialysed at pH 7.0, 4 degrees C and applied to a bacitracin-Sepharose column equilibrated in 0.05 mol/liter of tris-HCl pH 8.0. (J. Appl. Biochem., 1983 pp420-428). Material bound to the column was eluted using the
15 equilibration buffer supplemented with 1 mol/liter of NaCl and 10% (v/v) isopropanol. The fractions containing the enzyme activity sought were identified by incubation with the synthetic substrate Z-Pro-Pro as described in the Materials & Methods section. Fractions containing activity were then applied to a MonoQ column equilibrated in 0.025 mol/liter Tris-HCl pH 8.5. Under these conditions the enzyme bound to the
20 column and was eluted using an NaCl concentration gradient. Active fraction were pooled, dialysed and rechromatographed on a Mono Q column as described. The activity of the final concentrate measured on Z-Pro-Pro at 37 degrees C and pH 7.0 was 0.5 units/ml and 0.09 units/ ml at pH 5.5. This preparation has been used in the tests described in Example 11.

25 Only the enzyme isolated from *Xanthomonas campestris* pv *campestris* showed activity under acid conditions. A similar carboxypeptidase isolated from *X.rubrilineans* showed no activity at pH 5.5 and is, therefore, less suited for the applications anticipated.

30 Example 10

Isolation of a glycine-cleaving endoprotease from *Aspergillus oryzae*

35 An endoprotease that can cleave proteins rich in glycine residues has been identified in papaya extracts (glycyl endopeptidase, EC 3.4.22.25). However, this enzyme has several disadvantages for the application as envisaged because of its relatively high

(near neutral) pH optimum and high costs if produced in a pure form. Therefore the identification of an acid stable, glycin-specific endoprotease the enzyme in a potentially foodgrade microorganism could offer many advantages. To that end we have screened a number of commercially available foodgrade enzyme preparations.

5

The screening was carried out using the synthetic chromogenic peptide Z-Gly-Gly-pNA at pH 4.0 as the substrate. Six different enzyme preparations were tested and only Flavourzyme 1000 L (HPN 00011 from *Aspergillus oryzae*; NOVO, Denmark) showed some activity towards the synthetic substrate used. To isolate the enzyme responsible for this activity a large number of different purification protocols had to be tested. The following protocol proved to be successful.

First, the excess of small molecular weight components as present in the Flavourzyme material was removed by an extensive dialysis. During this dialysis step the Gly-specific activity dropped till approx 50% of its initial value. The resulting dialysed liquid was then further washed and concentrated using Amicon PM-10 ultrafiltration equipment. To the resulting liquid concentrate ammonium sulfate was added till 50% and after centrifugation more ammonium sulfate was added to the supernatant to reach 75% of the saturation level. The precipitate then obtained was collected by low speed centrifugation, dialysed and the resulting liquid was again concentrated using the PM-10 equipment. The concentrate was applied on a Sephadex G-100 gelfiltration column and the active fractions were pooled. The activity in each fraction was measured using Z-Gly-Gly-pNA at pH 4.0 as described in the Materials & Methods section. The pooled fractions were then subjected to ion exchange chromatography on HiTrap Q equilibrated in 0.05 mol/liter sodium acetate pH 5.0 and eluted with the same buffer containing 1 M NaCl. As before active fractions were identified by incubation with Z-Gly-Gly-pNA but now at pH 5.0, pooled and concentrated using the PM-10 equipment. The final concentrate if measured at pH 5.0 contained 0.008 units/ml. This solution was used to carry out the experiments as described in Example 11.

30 Example 11

Improved haze stability of 100% malt worts treated with selected auxiliary proteases

The present Example demonstrates that the anti-haze effect of endo-proA can be further improved by combining the EndoProA treatment with selected auxillary proteases. It is exemplified that a proline-specific carboxypeptidase from *Xanthomonas*

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campestris ("CarboxyPro") or a glycine-cleaving endoprotease from *Aspergillus oryzae* ("EndoGly") significantly reduce haze formation further when used in combination with endo-proA. The present Example focuses on haze reducing effects in 100% malt worts.

To prepare malt wort, 50 g of milled malt was mixed with 200 ml of water and heated to 50°C. Either 0 or 150µl of the EndoPro enzyme concentrate was added and the mash was subjected to a step-wise heating protocol as specified in Table 10. During this procedure the mash was stirred at 200 rpm. At the end, the mash was cooled to room temperature and water was added to compensate for any water evaporated. Finally the mash was filtered on paper to separate the wort (liquid) from the solids. The worts thus obtained were used to test the effects of the auxillary enzymes using the effect of PVPP as a reference.

Table 10. Mashing temperature protocol

Steps	Temperature	time
1	50°C	30 min
temperature increase	1°C/min	13 min
2	63°C	30 min
temperature increase	1°C/min	10 min
3	72°C	30 min
temperature increase	1°C/min	4 min
4	77°C	10 min

Because of its high affinity for polyphenol, PVPP is widely used in the industry to remove beer hazes caused by polyphenol-protein aggregates. In order to determine if CarboxyPro or EndoGly can have a haze reducing effect as PVPP, non-EndoPro treated worts were incubated with either 100µl CarboxyPro or 20µl EndoGly during 120 minutes at 50 degrees C after which the resulting haze stabilities were measured according to the procedure described in the Materials & Methods section. As a reference, non-EndoPro treated mash was incubated with PVPP for 15 minutes at room temperature prior to filtration and then measured. The results obtained are shown in Table 11.

Table 11: The chill haze effects of PVPP, CarboxyPro and EndoGly on a non-EndoPro treated 100% malt wort.

TRIALS n°	Enzyme active conditions			Controls	
	1	2	3	4	5
Enzyme added in the mash					
Endo-Pro (µl)	0	0	0	0	0
Enzymes added in the wort	120 min at 50°C				
Proline Carboxypeptidase (µl)	0	100	0	0	0
EndoGly (µl)	0	0	20	0	0
Wort haze control				PVPP is mixed 15 min prior paper filtration	
PVPP (Control - g/hl)				30	100
Haze measurement					
Wort Chill Haze test (EBC)	146	131	91.7	75.3	69.8
	144	134	92.7	79.0	68.1
Haze induced by the Chill Haze test (average - EBC)	145	132.5	92.2	77.2	69.0
Haze reduction (%)	0.0	8.6	36.4	46.8	52.4

EBC: nephelometric turbidity units recommended by the European Brewery Convention

5 According to the results shown in Table 11, incubations with both CarboxyPro and EndoGly result in worts that are less turbid upon cooling. However, the results also indicate that the addition of PVPP has a clearly superior effect.

10 A subsequent experiment was carried out in which a wort was used that had been pretreated with EndoPro in the mashing stage. In this experiment, the auxillary proteases were added to the wort in quantities of either 0, 100 or 500 microliters EndoGly or 0, 20 or 100 microliters CarboxyPro per 10 millilitres wort. All samples were then incubated for 120 min at 50°C after which the chill haze test was carried out. The results obtained are provided in Table 12.

Table 12: Wort chill haze results obtained after incubation of an EndoPro treated 100% malt wort followed by incubation with various amounts of CarboxyPro or EndoGly .

TRIALS n°	Enzyme active conditions					
	1	2	3	4	5	6
Enzyme added in the mash						
Endo-Pro (µl)	0	150	150	150	150	150
Enzymes added in the wort	120 min at 50°C					
Proline Carboxypeptidase (µl)	0	0	100	500	0	0
EndoGly (µl)	0	0	0	0	20	100
Haze measurement						
Wort Chill Haze test (EBC)	168	79.2	68.8	58.8	63.0	40.1
	168	80.6	70.0	57.7	64.8	36.0
Haze induced by the Chill Haze test (average - EBC)	168	79.9	69.4	58.3	63.9	38.1
Enzymes effect on Chill Haze Haze reduction (%)	0.0	52.4	58.7	65.3	62.0	77.4
CarboxyPro or EndoGly effect on EndoPro treated wort Haze reduction (%)		0.0	13.1	27.1	20.0	52.4

EBC: nephelometric turbidity units recommended by the European Brewery Convention

5 According to the results shown in Table 12, the combination of an EndoPro pretreatment with either CarboxyPro or EndoGly have significant effects on the haze stability of a wort. Depending upon the concentration of the auxillary proteases added effects can be obtained that are superior those obtained with PVPP in non-EndoPro treated wort (see Table 11).

10 Finally an experiment was carried out to test if the enzyme effects observed are the result of non-enzymic artefacts or specific proteolytic action. To that end 100 µl CarboxyPro or 20µl EndoGly were added to an EndoPro-treated wort. To prevent all enzymatic activities of the auxillary enzymes, the wort containing these enzymes was kept at 0 degrees C for 15 minutes (i.e. comparable with the incubation period used for PVPP) before measuring the wort chill haze formed. Wort treated with

15

various quantities of PVPP and then filtered was used as a reference.

Table 13: Effects of the incubation of EndoPro-treated 100% malt wort with CarboxyPro or EndoGly at 0 degrees C , or PVPP at room temperature

TRIALS n°	Controls				
	1	2	3	4	5
Enzyme added in the mash					
Endo-Pro (µl)	150	150	150	150	150
Enzymes added in the wort	enzymes added in non-active condition (at 0°C after the EndoPro treated wort was incubated 120 min at 50°C)				
Proline Carboxypeptidase (µl)	100	0	0	0	0
EndoGly (µl)	0	20	0	0	0
Wort haze control			PVPP is mixed 15 min with EndoPro treated wort previously incubated 120 min at 50°C. PVPP removal is performed by paper filtration		
PVPP (Control - g/hl)			30	100	500
Haze measurement					
Wort Chill Haze test (EBC)	77.5	82.2	66.3	59.3	52.3
Haze induced by the Chill Haze test (average - EBC)	77.5	82.2	65.9	60.6	53.5
Enzymes effect on Chill Haze Haze reduction (%)	53.9	51.1	66.1	60.0	52.9
Haze reduction (%)	3.0	-2.9	60.7	64.3	68.5
	3.0	-2.9	17.3	25.0	33.8

EBC: nephelometric turbidity units recommended by the European Brewery Convention

The data presented in Table 13 show that both CarboxyPro and EndoGly if incubated at 0 degrees have no effect which implies that their proteolytic activities are the direct cause for haze stabilisation observed in Tables 11 and 12. PVPP has a limited effect only (a haze reduction of a mere 17.3% versus a haze reduction of 46.8% under conditions described in Table 11). This observation re-confirms the impact an EndoPro incubation has on the total quantity of precipitable polyphenol-protein present: by preventing the formation of polyphenol-protein aggregates, the use of PVPP has almost become superfluous.

Example 12

Endo-proA has also endo-hydroxy-pro and endo-ala activity

It was investigated whether endo-proA also has endoprotease specificity for hydroxy proline and alanine. Therefore three synthetic peptides were synthesized covering the C-terminus of α_{s1} -casein B, with some modifications. The peptides were:

- 0139-59, SEKTTMPLW, the original C-terminus of α_{s1} -casein M=1091.5
- 0139-60, SEKTTMJLW, with J being hydroxy proline M=1107.5
- 0139-61, SEKTTMALW, with alanine substituted for proline M=1065.5

For analyzing such complex peptide mixtures so called *scan dependent MS/MS* is used. This method, in which each scan consists of three segments, is defined as follows:

- 1: *full scan* analysis,
- 2: *zoomscan* analysis for the determination of the charge state of the most intense ion in the full scan mass range,
- 3: *MS/MS* of the most intense ion in the full scan mass range to obtain amino acid sequence information.

All three peptides were dissolved in 0.1 % formic acid at a concentration of 75-100 $\mu\text{g/l}$ and were checked for their purity in LC/MS and LC/MS/MS mode using gradient elution. All three peptides could be identified by their protonated and doubly protonated molecules in LC/MS mode and in LC/MS/MS mode by total coverage of the amino acid sequence.

The synthetic peptides treated with endoproA were diluted 50 times before analysis. For LC/MS analysis of the peptides formed after treatment, the gradient has to be adapted slightly. If indeed endoproA has proline and hydroxy proline specific specificity the peptides should be cleaved in the following parts:

5

SEKTTMP and LW, with protonated molecules at m/z 793.4 and 318.1, respectively. SEKTTMJ and LW, with protonated molecules at m/z 809.4, and 318.1, respectively.

10 Both predicted peptide masses could indeed be observed in the ion chromatograms of the endopro treated peptides, and were checked in LC/MS/MS mode having the correct amino acid sequence.

The third synthetic peptide also showed the same pattern by observing m/z 767.4 of SEKTTMA and m/z 318.1 of LW in the ion chromatogram. Endopro preferentially cleaves C-terminal at P, J and A, but also peptides being 1,2, or 15 3 amino acids shorter could be observed and unequivocally be identified with LC/MS/MS. However these percentages were all below 6 %. An overview of the above is given in table 14.

20 Table 14: peptide formed for all three synthetic peptides after treatment with endoproA. Amino acid sequences were checked by their protonated molecule and MS/MS characteristic.

peptide formed	intensity normalized		
	SEKTTMPLW	SEKTTMJLW	SEKTTMALW
SEKT		0,43	0,33
SEKTT	0,00	0,37	0,33
SEKTTM	3,40	3,11	5,56
SEKTTMP	100,00		
SEKTTMJ		100,00	
SEKTTMA			100,00

25 Conclusion

Indeed endoproA has hydroxy proline and alanine specificity, besides proline specificity.

Example 13

PH optimum for endo-pro.

5 DNA sequences according to SEQ ID NO: 1 and SEQ ID NO: 3 were expressed in
Aspergillus niger iso 502. This resulted in polypeptides according to SEQ ID NO: 5 and
SEQ ID NO: 4 respectively. These polypeptides will be further referred to as endopro
RUS and endopro GAM respectively. Both enzymes were found to be very much alike
but were also found to differ in some biological aspects. Endopro RUS had a higher
10 specific activity and was found somewhat more active in removing haze in beer.

1) pH optimum Endopro:

Proteolytic activity of Endopro's were measured with prescription of Roche albeit
that the incubation buffer is replaced by others, in a pH range of pH 4.5 – 4.8 – 5.0 – 5.5 –
6.0 – 7.0 – 8.0. 0.1 M Tris/HCl buffers were used for pH 7 and 8. For the lower pH's 0.1 M
15 Hac buffers were used. The buffers contained 0.02 M CaCl₂.

Reactions were performed at 50 °C. See Table 15 and 16

EndoproRUS (6 U/mL) and EndoproGAM (ca. 4 U/mL) were diluted to 0.5 U/mL. 10
µL sample was added in the incubation mixture and volume compensated with the
incubation buffer.

20 2) T-optimum Endopro

Reactions performed at 40 – 50 – 55 and 60 °C and at pH 5.0. Samples were taken
after 0.5 – 1 – 2 – 5 and 20 hours.

EndoproRUS and EndoproGAM were diluted to 0.5 U/mL. 10 µL sample was added
in the incubation mixture and volume compensated with the incubation buffer.

25 See Tables 17 and 18

3) Freeze/thawing

It was speculated that freezing and thawing enhanced haze formation in the
endoproGAM solution. Purified EndoproGAM was ultracentrifugated in order to make it
clear. The almost clear supernatant was freeze (- 26 °C)/ thawed for five times. Before and
30 after the treatment turbidity and activity were measured.

4) Stability

RUS and GAM samples diluted to 3 U/mL and at pH 5.0 (was already eluent pH). NaN₃ was added (0.01 % w/v). The solutions were stored at 35 °C. Also a sample of turbid concentrated GAM ultrafiltrate was stored. The sample was centrifuged in Sorvall for 40 min at 35,000 g (results in cell wall pellet, fluffy pellet, fatty layer, turbid intermediate layer; the intermediate layer was further used).

5

Table 15 pH optimum

pH	RUS		GAM	
	E ₅₇₄	Rel. %	E ₅₇₄	Rel. %
4.5	0.227	30.2	0.225	24.0
4.8	0.228	38.3	0.278	29.6
5.0	0.521	69.4	0.554	59.0
5.5	0.751	100.0	0.939	100.0
6.0	0.675	89.9	0.870	92.7
7.0	0.399	53.1	0.627	66.8
8.0	0.011	1.5	0.313	33.3

Table 16 T-optimum

t hours	RUS				GAM			
	4	5	5	6	4	5	5	6
	0 °C	0 °C	5 °C	0 °C	0 °C	0 °C	5 °C	0 °C
0	100	100	100	100	100	100	100	100
0.5	93	102	100	67	98	94	94	84
1	105	98	98	81	102	97	92	83
2	99	102	96	74	103	95	94	79
5	99	99	88	67	95	88	92	80
22	104	90	66	33	105	88	82	64

10

Freeze / thawing Endopro GAM

After five times freezing/thawing the turbidity at 520 nm before / after

Before : 0.331 After : 0.355

Activity was determined by means of Z-Gly-Pro-pNA substrate

Before : 0.156 U/mL After : 0.136 U/mL

5

Stability

NaN₃ does not affect the activity. Endopro is not inhibited by Pefablock or inhibitor Complete cocktails.

Turbidity at E 520 :RUS and GAM (clarified by ultra centrifugation) :

10

	E ₅₂₀
EndoproRUS	0.035
EndoproGAM	0.361
EndoproGAM (after 48 hours- 35 °C)	0.725

15

After 5 min – 16,000g (Eppendorf) turbid GAM sample totally clear !! Also the concentrated UF broth is completely clear after 35 °C storage for 24 hours and Eppendorf centrifugation!

Sample	dilution	E405/ min 2 days	E405/ min 25 days	E280 (1:500)
GAM UF broth	20 x	0.046		0.341
GAM UF broth – 35 °C	20x	0.049	0.051	0.363
GAM UF broth – 35 °C – Eppend. sup	20x	0.050	0.074	0.211
GAM UF broth – Sorvall sup – 35 °C – Epp. Sup	20x	0.047	0.054	0.197
GAM 3 U/mL	30x	0.100	0.090	0.343
RUS 3 U/mL	30x	0.112	0.103	0.310
RUS 6 U/mL	30x	0.196		0.573
GAM 4 U/mL	30x	0.137		0.401

Specific activity.:

5 Calculation example: GAM 3 U/mL

$$\square_{405} = 10,500 \square 0.0105 \mu\text{M} \square 0.0105 \text{nmol/mL}$$

$$\square_{\text{E/min}} = 0.100 \quad 0.100 / 0.0105 = 9.52 \text{ nmol/mL} \rightarrow 1.35 = 12.85 \text{ nmol/mL}$$

$$12.85 \text{ in } 100 \mu\text{L} \square 128.5 \text{ nmol / mL} = \mu\text{M. Dilution } 30 \times \rightarrow 3850 \mu\text{mol/L} = 3850 \text{ U/L}$$

10 $E_{280} (1:500) = 0.343 \rightarrow \text{undiluted } 171.5 \quad \square_{280} = 3 \Rightarrow 171.5 / 3 = 56.98 \text{ g/L}$

$$\text{Spec. Act. GAM: } 3850 / 56.98 = 68 \text{ U/g} \square 0.068 \text{ U/mg}$$

Conclusions

- pH Optima of both the enzymes for CN-resorufin are the same pH 5.5 (at pH 5.0 flocculation of substrate occurs !). However EndoproGAM still has 40 % rest activity at pH 8.0 (see Fig 2).

- EndoproGAM seems to be more stable than EndoproRUS.
- Freeze / thawing has only a minor effect, if any, on turbidity and stability on EndoproGAM.
- 5 • Flocculating / aggregating material is easily removed by centrifugation after a 35 °C heat treatment for 48 hours. Probably heating time may be reduced at higher incubation temperature. Precipitability of aggregating material is highly enlarged by this heat treatment. Aggregation is a one time event. In this way the turbidity problem in EndoproGAM broth may be tackled.
- 10 • Both Endopro GAM / RUS in solution do not lose activity after 25 days storage at 35 °C.
- Specific activity of EndoproRUS is higher than for EndoproGAM.

ABSTRACT

The invention relates to a method for the prevention or reduction of haze in a beverage by the addition of a prolyl-specific and/or hydroxyprolyl specific and/or alanine specific endoprotease and to new beverages obtainable by the method according to the invention. It also relates to new endoproteases. It also relates to methods as described above wherein auxiliary enzymes are used in combination with the specific endoproteases. Sequence information of a genomic DNA, cDNA aswell as protein sequenses are provided

CLAIMS

1. Method for the prevention or reduction of haze in a beverage wherein a proline-specific and/or hydroxy-prolyl-specific endoprotease and/or an alanine-specific endoprotease is added to the beverage.
2. Method according to claim 1 wherein the endoproteases are in an essentially isolated form
3. Method according to any one of claims 1 - 2 wherein an endoprotease is added having a maximum specific activity at a pH which corresponds to the pH of the beverage it is added to.
4. Method according to any one of claims 1 - 3 wherein the beverage contains proteins
5. Method according to any one of claims 1 - 4 wherein the beverage contains polyphenols
6. Method according to any one of claims 1 - 5, wherein the beverage has a pH value at or below 7.0, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5 or 3.0.
7. A method according to any one of claims 4 - 6 wherein at least 150 milli-units of specific endoprotease activity, as determined by an activity measurements using Z-Gly-Pro-pNA, Z-Gly-hydroxy-pro-pNA or Z-Gly-Ala-pNA as a substrate, is added to the beverage per gram protein in the beverage.
8. A method according to any one of claims 4 - 6 wherein at least 500 milli-units of specific endoprotease activity, as determined by an activity measurements using Z-Gly-Pro-pNA, Z-Gly-hydroxy-pro-pNA or Z-Gly-Ala-pNA as a substrate, is added to the beverage per gram protein in the beverage.
9. A method according to any one of claims 4 - 6 wherein at least 1 unit of specific endoprotease activity, as determined by an activity measurements using Z-Gly-Pro-pNA, Z-Gly-hydroxy-pro-pNA or Z-Gly-Ala-pNA as a substrate, is added to the beverage per gram protein in the beverage.
10. Method according to any one of claims 1 - 9 wherein the beverage is a liquid used in the production of beer.

11. Method according to any one of claims 1 - 9 wherein the beverage is a liquid used in the production of wine.
12. Method according to any one of claims 1 - 9 wherein the beverage is a liquid used in the production of fruit juice.
13. Method according to claim 10 wherein a prolyl-specific endoprotease is added to a mash.
14. Method according to claim 10 wherein a prolyl-specific endoprotease is added to a beer before haze is formed
15. Method according to claim 10 wherein a prolyl-specific endoprotease is added to a fermented beer after haze has been formed.
16. Method according to claim 11 wherein a prolyl-specific endoprotease is added to a fermented wine.
17. Method according to any of claims 1 to 16 wherein an auxiliary enzyme is added to the beverage in order to further reduce or prevent haze formation.
18. Method according to claim 17 wherein the auxiliary protein is a purified exoprotease or endoprotease.
19. Method according to claims 17 or 18 wherein the exoprotease is a proline-specific carboxypeptidase.
20. Method according to claim 19 wherein the proline specific carboxypeptidase is obtainable from *Xanthomonas*.
21. Method according to claims 17 or 18 wherein the endoprotease is a glycine-specific endoprotease or an aspartic acid protease.
22. Method according to claim 21 wherein the aspartic protease is Fromase ®
23. An isolated polypeptide having prolyl-specific and/or hydroxyprolyl-specific and/or alanine-specific endoprotease activity with an acidic pH optimum.
24. Polypeptide according to claim 23 wherein the pH optimum lies at or around pH 5.5.
25. Use of a specific endoprotease according to any of claims 23 or 24 in the preparation of a beverage

26. Use of purified prolyl-specific and/or hydroxyprolyl-specific and/or alanine-specific endoprotease in the preparation of beer, wine or fruit juice.
27. Beverage obtainable by a method according to any one of claims 1 – 22 and/or 25 – 26.
28. Beer obtainable by a method according to claim 10.
29. Wine obtainable by a method according to claim 11.
30. Fruit juice obtainable by a method according to claim 12

Figures
Figure 1

Fig 1

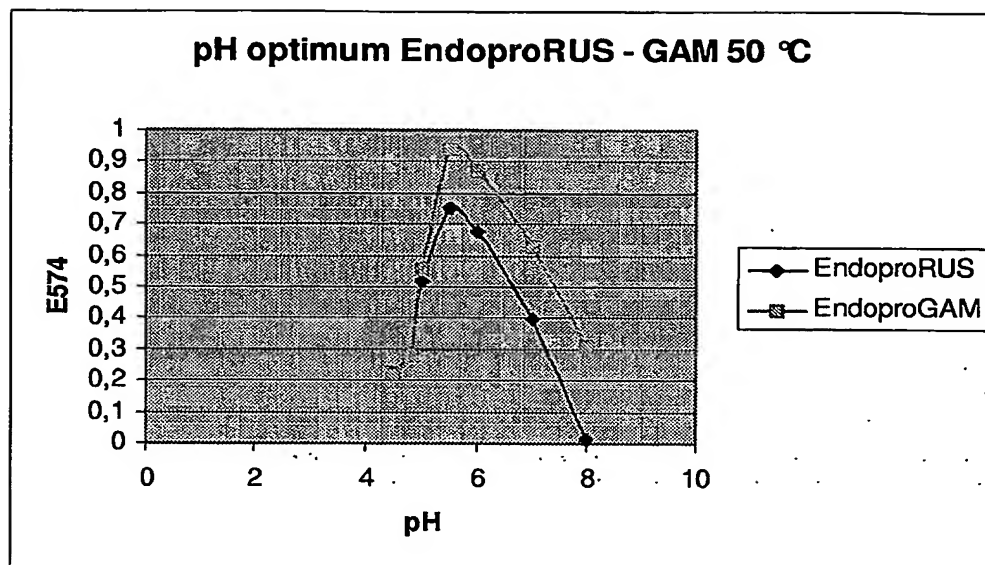


Figure 2

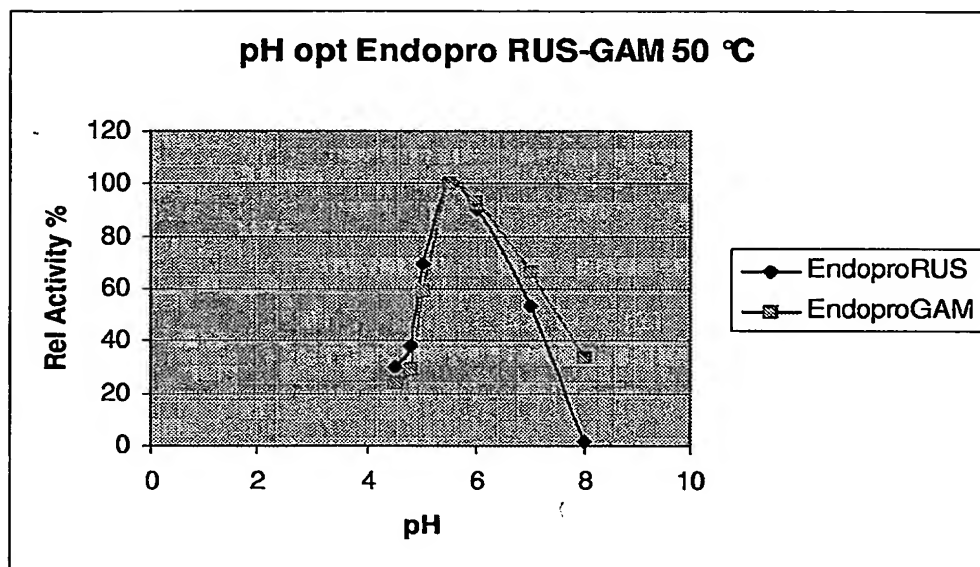


Figure 3

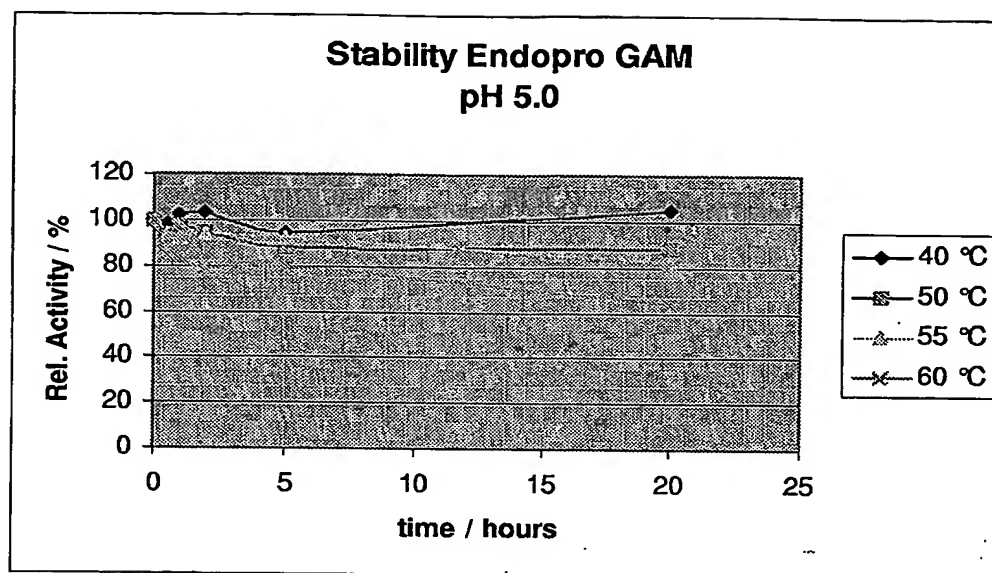


Figure 4

